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A classification system for gonad development in triploid *Crassostrea virginica*

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ABSTRACT

Triploidy is a common form of genetic improvement that confers benefits associated with reduced fecundity. Triploid oysters produce fewer gametes yet undergo gametogenesis and develop gonads to variable extents. The variable and typically abnormal gonad development in triploid oysters has often been summarized using criteria developed for the more uniform development of diploid oysters, which can lead to misleading characterizations. Classification systems designed for triploid oysters, such as that for triploid *Crassostrea gigas*, have allowed for less subjectivity, more repeatability, and have engendered hypotheses of developmental pathways specific to triploids. Despite recent interest in gametogenesis of triploid *Crassostrea virginica* in connection with mortality events, gonad development in triploid *C. virginica* has primarily been summarized using criteria developed for diploid oysters. In this work, a novel classification system was developed for gonad development in triploid *C. virginica* while examining triploids and diploids sampled regularly from a site with and a site without a "triploid mortality" event. Triploids were classified based on the type of gonia present, presence of spermatogenic cells or oocytes, and relative abundance of gametes. The system developed for triploid *C. virginica* was in stark contrast to the previously characterized system for triploid *C. gigas*. A relationship between the nature of the gonia and fecundity, as described for triploid *C. gigas*, was absent, and instead, we hypothesize that the nature of the gonia indicates sex. Regular, "normal" gonia were associated with male triploids, whereas irregular gonia indicated female lineages. Pathways for gonad development in triploid *C. virginica* are proposed based on anatomical observations and time series data. Gonads were mostly similar for triploids at the affected (exhibiting triploid mortality) site and control site as well as between moribund and live triploids sampled during the mortality event. The specific anatomy of gonad development appears unlinked to triploid mortality; however, underlying metabolic processes during gametogenesis remain the leading culprit.

1. Introduction

Polyploidy, the condition of having three or more chromosome sets, is a widely applied method of genetic improvement in oyster aquaculture. Originally, polyploids were induced by suppressing cell division during meiosis (reviews by Beaumont and Fairbrother, 1991; Thorgaard, 1986) and had nominal utility for fish and shellfish. Now, for oysters, the most common method to produce commercial triploids is through tetraploid X diploid crosses (reviews by Guo et al., 2009; Piferrer et al., 2009). The manifestation of triploidy is reproductive sterility. In theory, a sterile fish or shellfish avoids poor performance associated with sexual development, such as slower growth, reduced flesh quality, and higher mortality (Lincoln et al., 1974; Refstie et al., 1977; Stanley et al., 1981).

Triploid oysters have become a popular commercial product because expected benefits have been realized, including faster growth (e.g. Dégremont et al., 2012; Nell and Perkins, 2005), and consistent meat weight during the spawning season (e.g. Allen and Downing, 1986; Matt et al., 2020). A major portion of hatchery-based aquaculture production around the world is now dedicated to triploids, including triploid *Crassostrea gigas* on the West Coast of the US, western Europe, Australia, and China, and triploid *Crassostrea virginica* on the East Coast and Gulf Coast of the US. In the Chesapeake Bay, triploid *C. virginica* have become especially popular. Triploids have comprised 85% of the oysters planted in the Virginia portion of the Chesapeake Bay over the last few years, making up most of the approximately 35 million cultured oysters sold each year (Hudson, 2018).

The added value of triploid oysters is principally due to their reduced fecundity, a trait that varies by species and method of production. In *C. virginica*, *C. gigas*, and *Saccostrea commercialis*, triploids

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produced by inhibiting polar body II in newly fertilized eggs, or "chemical" triploids, typically produce relatively few oocytes and no spermatozoa (Allen and Downing, 1990; Barber and Mann, 1991; Cox et al., 1996; Gardner et al., 1994; Lee, 1988; c.f Normand et al., 2008). Gamete production of triploids produced by mating tetraploids to diploids, or "mated" triploids, has also been evaluated in *C. gigas* (Hermabessiere et al., 2016; Jeung et al., 2016; Jouaux et al., 2010, 2013; Suquet et al., 2016) and *C. virginica* (Guévélou et al., 2019; Peachey and Allen, 2016; Wadsworth et al., 2019). In contrast with most findings in chemical triploids, mated triploid *C. gigas* commonly produce spermatozoa (Jouaux et al., 2010; Suquet et al., 2016), yet it is less clear if female chemical triploids are more or less fecund than mated triploids (Gong et al., 2004; Suquet et al., 2016). Gamete production in mated triploid *C. virginica* has been reported to resemble chemical triploid *C. virginica*, consisting of highly reduced numbers of oocytes and an absence of spermatozoa (mated: Guévélou et al., 2019; Wadsworth et al., 2019; chemical: Lee, 1988; Barber and Mann, 1991). Anecdotally, for the sake of obtaining eggs for tetraploid induction of *C. virginica*, mated triploids have been more fecund than chemical triploids (S. Allen, VIMS, unpubl.; J. Supan, Louisiana State University, unpubl.).

Gonad development in triploid oysters has been shown to contrast starkly with development in diploids. Many authors have described sexual maturation in triploids to be retarded (Allen and Downing, 1990; Cox et al., 1996; Gardner et al., 1994; Guévélou et al., 2019; Normand et al., 2008; Wadsworth et al., 2019) compared to diploids. Additionally, while sexual maturity in diploids is rather uniform, triploids vary widely among themselves in both the extent of gonadal follicle development and production of gametes. Allen and Downing (1990) observed a range of gonadal follicle development among triploids, from extensive to severely reduced, as well as high variation in numbers of oocytes and spermatids. Similarly, Normand et al. (2008) reported especially high inter-individual variation in extent of gonad development in triploid *C. gigas* at the time diploids were sexually mature.

Gonad development in triploids often has been evaluated by using developmental stages created for diploids (Allen and Downing, 1990; Guévélou et al., 2019; Normand et al., 2008; Wadsworth et al., 2019); however, several authors have created classification systems specific to triploid development. Gardner et al. (1994) and Cox et al. (1996) developed similar classification systems for triploid *C. gigas* and triploid *S. commercialis*, citing that existing systems for diploids were unsuitable. Collectively, the systems of Gardner et al. (1994) and Cox et al. (1996) were based on presence or absence of spermatids for males and the relative maturation state and number of oocytes for females. Later, Jouaux et al. (2010) developed a more comprehensive classification system for triploid *C. gigas* and suggested two major pathways of development: triploids with abnormal, disturbed gametogenesis, referred to as β triploids, and those with diploid-like gametogenesis, referred to as α triploids. The α and β triploids could be distinguished early in development simply by the nature of the germ cells, or gonia, that give rise to gametes. Abnormal gonia indicated β triploids, whereas normal gonia indicated α development (Jouaux et al., 2010).

Classification systems developed for triploid oysters are an important improvement in understanding the nature of gametogenesis in triploids. For one, a more suitable system makes classification less subjective and more repeatable, as applications of diploid criteria often involve vague, study-specific descriptions. For example, Guévélou et al. (2019) used traditional criteria developed for diploids (Kennedy and Krantz, 1982; Loosanoff, 1942) to assess gonad development in diploid and triploid *C. virginica*, yet also classified triploids with highly reduced gonad development as "ripe" because it was the observed culmination of development for triploids in their study. Perhaps more importantly, a triploid-specific classification system can result in more meaningful dissection of sterility as a trait in triploids. The pathways of development proposed by Jouaux et al. (2010) led to the bifurcation of α and β categories, which in turn impelled Dheilly et al. (2014) to look for differences in gene regulation between α and β triploid *C. gigas*.

A Jouaux-like classification system for gonad development in triploid *C. virginica* does not exist, despite recent interest in gametogenesis of triploid *C. virginica*. Gametogenesis has been a focus of unusual mortality in triploid *C. virginica* associated with late spring conditions, or "triploid mortality" (Guévélou et al., 2019; Matt et al., 2020). Reports from oyster farms in the Chesapeake Bay, USA, since 2012, as well as empirical studies (Guévélou et al., 2019; Matt et al., 2020) have been involved in defining triploid mortality as death of near-market sized (76 mm) triploids in late spring. The timing of the events implicates gametogenesis as factor in the mortalities, because gametogenesis is well underway in *C. virginica* in late spring in the Chesapeake Bay (Kennedy and Krantz, 1982). A connection between gonad development and differential mortality in triploid *C. virginica* has been previously investigated in the Chesapeake Bay (Guévélou et al., 2019) and the Gulf of Mexico (Wadsworth et al., 2019), mostly using criteria designed for gonad development in diploids.

The primary objective of this study was to develop a more precise description of the stages and extent of gonad development in triploid *C. virginica* and to apply this "objective" index to the question of triploid mortality. Histological cross sections for this investigation were available from a recent study on triploid mortality (Matt et al., 2020), and thus an association between gonad development and triploid mortality could be examined. Samples of triploids and diploids were available from February 2016 to August 2016 from a site with and without a triploid mortality event.

2. Methods

Oysters in this study were the same individuals as those sampled during a field trial described in Matt et al. (2020). An abbreviated version of the methods involving brood stock, spawns, deployment, and ploidy verification are presented here.

2.1. Brood stock and crosses

Triploid and diploid *C. virginica* were simultaneously produced at the research hatchery of the Aquaculture Genetics and Breeding Technology Center (ABC) located at the Virginia Institute of Marine Science (VIMS) in Gloucester Point, Virginia in February of 2015. The diploid brood stock consisted of the ABC DEBY line (Ragone Calvo et al., 2003) and a proprietary commercial line from Mook Sea Farms, Walpole, Maine. The tetraploid brood stock consisted of ABC's GEN and VBOY lines. The GEN line has been reared in the Chesapeake Bay and propagated by ABC since 2003, while the VBOY line has partial parentage from Louisiana. A pool of sperm from males and a pool of eggs from female diploids from the ABC DEBY line (chromosome set contribution: V) were crossed to produce the reference diploid oysters (VV). The same pool of V eggs, as well as a pool of eggs from the Maine diploids (chromosome set contribution: M), were crossed to male tetraploid oysters from GEN (chromosome set contribution: VV) and VBOY (chromosome set contribution: LL) in a 2 × 2 matrix to produce triploid crosses (VVV, VVM, LLV, and LLM).

2.2. Sites and experimental deployment

Oysters were deployed to three field sites in June 2015. Two sites were commercial farms on the bayside of the Eastern Shore of Virginia and the other was on the western side of the Chesapeake Bay. For the eastern sites – Nandua Creek (ND) and Occohannock Creek (OC) – triploid mortality had been observed in 2014. For the western site – Rappahannock River (RR) – no triploid mortality has been observed. Deployment of the experiment took place between February 29 and March 3 of 2016. From each cross at each site, 450 oysters were haphazardly selected and equally split into 3 bags. Oysters were reared in single-tier bottom cages in the subtidal zone.

2.3. Ploidy verification

Oysters were sampled twice to verify ploidy via flow cytometry (FCM) (Allen and Bushek, 1992). Twenty-five oysters from each cross were verified prior to field deployment (April 2015) and 15 oysters were verified during the initial field sampling (February/March 2016).

2.4. Site visits and sampling

Sites were visited once or twice a month from April to August of 2016. Live oysters were sampled, without replacement, at experimental deployment and during all site visits in the spring and summer (April-August). For all sampling times after deployment, live oysters were randomly sampled. Five live oysters from each bag were selected, except in May and June when seven live oysters from each bag of triploid oysters were selected. Oysters were considered moribund if the shell commissure was only partially sealed. All moribund oysters were sampled.

2.5. Histology

From all samples (live and moribund), a 4 mm section of tissue was cut perpendicular to the anterior–posterior axis, slightly ventral of the labial palps. For live samples, tissue sections were weighed. All tissue sections were fixed in Davidson's solution for 48 h, then stored in 70% ethanol.

All samples of VV, VVV, VVM, LLV, and LLM from ND and RR were processed for histology. Additionally, samples of VV from OC in April and May were selected to provide another diploid comparison for ND and RR. Moribund oysters sampled during the middle of the mortality event at ND (May 24) were also processed for comparison with live oysters.

Samples were processed for histology by standard methods used at the VIMS Shellfish Pathology Lab (Carnegie and Burreson, 2011). Sections were dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

2.6. Gonad development

Hematoxylin and eosin stained slides were examined with a Nikon Eclipse E200 compound microscope. Transverse sections were evaluated for follicle and gamete development between the mantle and digestive gland.

Diploids were assigned a stage of gametogenic development, similar to that used by Kennedy and Krantz (1982) for *C. virginica*, Allen et al. (1986) for *Mya arenaria*, and Allen and Downing (1990) for *C. gigas*. The classification system involved the following stages: Inactive (I), Very Early Active (VEA), Early Active (EA), Active (A), Late Active (LA), Ripe (R), Spawning (S), Advanced Spawning (AS), and Spawned Out (SO). Stages were distinguished based on morphology of the follicles, follicle contents, and a visual estimate of the percentage of the incipient gonad area occupied by gonadal follicles, or follicle coverage. The incipient gonad area was defined as the area between the digestive tissue and mantle. A full description of these stages is in the appendix (Table A.1). Diploids were sometimes assigned to intermediate stages (i.e., EA-A or A-LA). Diploids were classified female if oocytes were present, male if spermatogenic cells (spermatogonia, spermatocytes, spermatids, or spermatozoa) were present, hermaphrodites if oocytes and spermatogenic cells were present, and unidentifiable if neither oocytes nor spermatogenic cells were present.

Gonad development was expected to be abnormal in triploid oysters. Therefore, several aspects of gonad development were examined in further detail among the triploid oysters. These additional features were the following: morphology of the gonia, extent of follicle development, and the type and relative abundance of cells in the follicles.

2.7. Statistical analysis

Statistical analysis was used to compare gonad development between live and moribund triploids. A Chi-squared Test of Independence was used to determine if gonad development was independent of status (live or moribund) at $\alpha = 0.05$. All graphing and statistical analyses were done in R (R Core Team, 2019).

3. Results

3.1. Ploidy verification

Results of ploidy verification were reported in Matt et al. (2020). All samples from putative diploid crosses were diploid, and all samples from putative triploid crosses were triploid, except 1 of the 25 oysters in the VVM group sampled in April and 1 of the 15 oysters in the LLM group sampled from RR in March, which were diploid. These individuals were discarded and left out of analyses.

3.2. Mortality

Mortality results are reported in more detail in Matt et al. (2020). In brief, substantial mortality only occurred in triploids at ND during late spring. Between April 14 and June 7 at ND, cumulative mortality increased from 0% in all crosses to 27% in VVV, 22% in VVM, 17% in LLV, 8% in LLM, and 1% in VV. Between June 7 and August 9 at ND, all crosses incurred low mortality: VVV (3%), VVM (6%), LLV (4%), LLM (9%), VV (1%). Cumulative mortality was $< 10\%$ at OC and $< 12\%$ at RR through August. Thus, only ND displayed the classic "triploid mortality" of *C. virginica*.

3.3. Gonad development in diploids

The diploids (VV) at ND consistently had more advanced gonad development than diploids at RR during the winter and spring (Fig. 1a). In February/March, most diploids at ND were Early Active, while most at RR were Very Early Active. Most diploids at ND were Ripe (47%) in early May, while none were Ripe at RR. By late May, nearly all diploids at ND were Ripe (80%) or Spawning (13%), whereas only 27% were Ripe and 0% were Spawning at RR. In early June, nearly all diploids at ND had spawned (93%) compared to just 53% at RR (Fig. 1b).

Diploids at ND and RR had mostly similar gonad development during the summer (Fig. 1b). The exceptions were some diploids at ND that were Ripe in July (33%) and Late Active in August (13%) (Fig. 1b), perhaps having recycled.

Samples of diploids (VV) from OC in April and late May provided an additional comparison for gonad development in diploids. Diploids at OC had more advanced gonad development than diploids at RR (Fig. 2a) and were slightly behind diploids at ND (Fig. 2b). In April, the majority at OC were Active (40%), whereas diploids at RR were mostly Early Active (67%). A greater percentage were Active, Active to Late Active, or Late Active at ND (80%) than at OC (53%) in April. By late May, nearly all diploids at OC were Ripe (47%) or nearly Ripe (47%), while most diploids at RR were Late Active (53%) and only 27% were Ripe. Nearly all diploids were ripe at ND by late May (80%).

Sex ratio was similar among diploids at ND, RR, and OC. Excluding the unidentifiable diploid oysters from February/March, 63% at ND and 65% at RR were female, and in April and May, 63%, 70%, and 77% of diploid oysters at ND, RR, and OC were female, respectively (Figs. 1, 2).

Sex ratio shifted in late summer in diploids at ND and RR. Females were the majority at ND and RR for most sampling times from April to July. In August, most diploid oysters were male at ND (53%) and RR (60%) (Fig. 1).

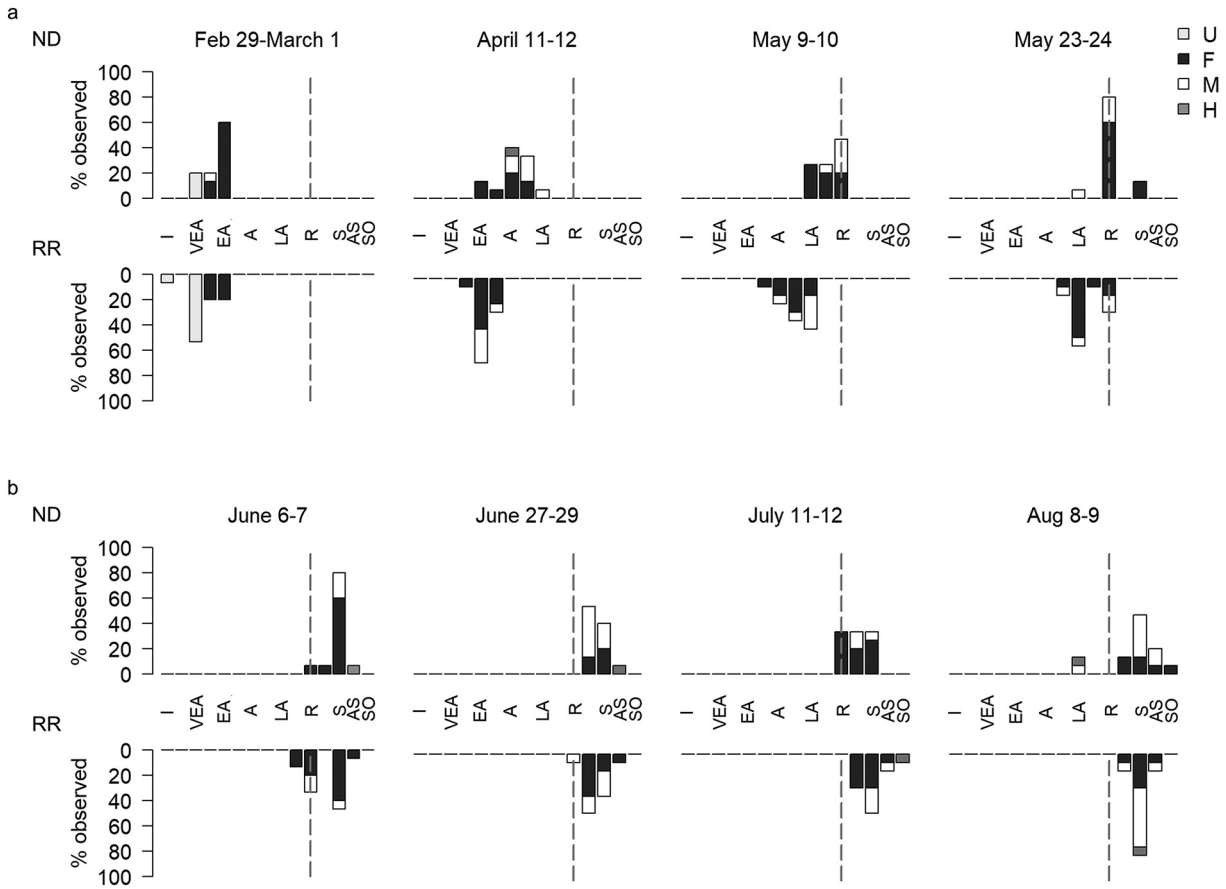

Fig. 1. Percentage of diploid *Crassostrea virginica* individuals of each gametogenic stage sampled from Nandua Creek (top) and Rappahannock River (bottom). a. Four time periods in winter and spring of 2016: February 29–March 1, April 11–12, May 9–10, and May 23–24. b. Four time periods in late spring and summer of 2016: June 6–7, June 27–29, July 11–12, and August 8–9. Dotted line represents mature gametogenic development (ripe stage). ND: Nandua Creek; RR: Rapphannock River; I: Inactive; VEA: Very Early Active; EA: Early Active; A: Active; LA: Late Active; R: Ripe; S: Spawning; AS: Advanced Spawning; SO: Spawned Out; U: Unidentifiable; F: Female; M: Male; H: Hermaphrodite.

3.4. Observations of triploid gametogenesis

Gonad development was abnormal in triploids, and the most common abnormalities involved the gonia. In some triploids, follicles contained gonia of uniform size and shape. The nuclei of these gonia ranged from slightly basophilic to slightly acidophilic, and they often contained a conspicuous nucleolus. Gonia fitting this description were referred to as α gonia, and their presence was associated with early stages of spermatogenesis. Typically, α gonia existed in one or two layers along the follicle wall, immediately adjacent to primary spermatocytes (Fig. 3).

A population of abnormal gonia, referred to as β gonia, were present in other triploids. β gonia were almost always present in multiple layers along the follicle wall (Fig. 4). The size of nuclei in β gonia was more variable than in α gonia, and the nuclei in β gonia were typically larger and more acidophilic. Often, a significant portion of the nucleus in β

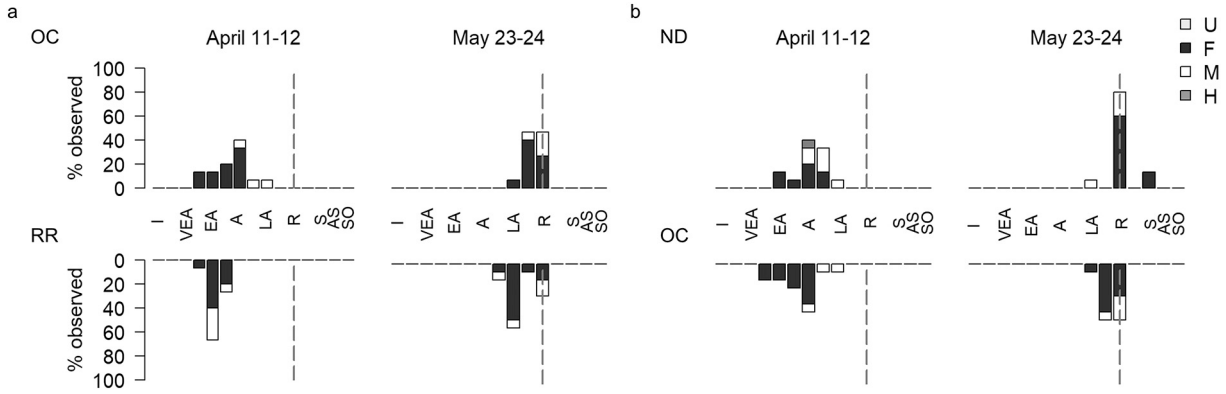

Fig. 2. Percentage of diploid *Crassostrea virginica* individuals of each gametogenic stage sampled from sites in the Chesapeake Bay during April 11–12 and May 23–24 of 2016. a. Occohannock Creek (top) and Rappahannock River (bottom). b. Nandua Creek (top) and Occohannock Creek (bottom). ND: Nandua Creek; RR: Rapphannock River; OC: Occohannock Creek; I: Inactive; VEA: Very Early Active; EA: Early Active; A: Active; LA: Late Active; R: Ripe; S: Spawning; AS: Advanced Spawning; SO: Spawned Out; U: Unidentifiable; F: Female; M: Male; H: Hermaphrodite.

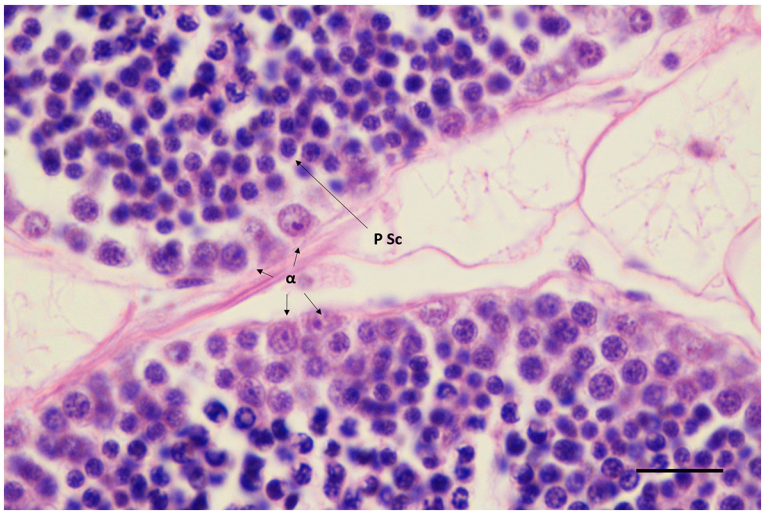

Fig. 3. Gonadal follicles of triploid *Crassostrea virginica* containing α gonia (α) and primary spermatocytes (P Sc). Scale bar = 10 μm. Magnification = 400×.

gonia was completely void, juxtaposed by a darkly staining region in the remainder of the nucleus (Fig. 4). Many nuclei in β gonia also contained condensed chromosomes, evident as bulky, rod-shaped, basophilic structures (Fig. 4). Nuclei in β gonia were also often completely opaque, and in many of these cases, it appeared the nucleus had retracted (Fig. 4).

Overall, follicle development was retarded and more variable in triploids compared with diploids. For diploids, follicles grew throughout the gonad over time, resulting in near total occupation of body tissue by late spring or early summer. A similar overall progression in follicle development was observed in triploids; however, this development progressed more slowly. High variance in the extent of follicle development existed among triploids throughout the spring and summer. For example, in mid-summer, while most triploids had significant follicle development, a small portion (approximately 15%) still had rudimentary, unbranched follicles.

Gametes in triploids were typically absent in February/March. In late spring and summer, 12% of triploids produced substantial numbers of observable oocytes, i.e., more than one egg per follicle, while in 34% of triploids, gametes were absent. Most common were triploids that had a few oocytes dispersed within the entire histological cross section, some follicles with and some without gametes.

The type and number of cells in the gonad were variable among triploids undergoing spermatogenesis. Triploids undergoing spermatogenesis often had follicles containing abundant primary spermatocytes. Usually in these cases, a much smaller number of spermatids was found near the center of the follicle. Except for one triploid (out of 913 examined) that resembled a ripe diploid male, a population of cells resembling secondary spermatocytes was absent in triploids. A high ratio of spermatids to primary spermatocytes was rare, as was the presence of spermatozoa. Overall, only 17% of triploids with evidence of spermatogenesis had spermatozoa.

Some triploids showed evidence of spawning. Regarding spawning, the only criteria applicable to triploids from the diploid classification system were the contents and morphology of the genetical canals or ducts (Kennedy and Battle, 1964) located near the mantle edge of the gonad. Other criteria used to assess if diploids spawned, such as irregular arrangement of follicles, shrunken follicles, or partially empty follicles, were impossible to apply to triploids because of their abnormal development. Triploids were considered to have spawned if the ducts contained a substantial number of oocytes or spermatogenic cells and the cells appeared to be entering the ducts from neighboring interconnected follicles. Overall, 34 triploids were considered to have spawned, and most (29) were from the August sampling. Due to lack of

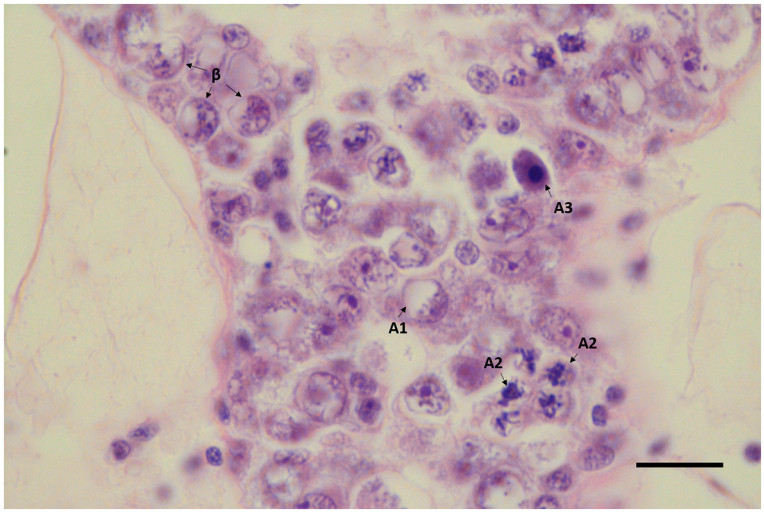

Fig. 4. Gonadal follicles of triploid *Crassostrea virginica* containing β gonia (β), of which some have nuclei with void areas (A1), condensed chromosomes (A2), or opaque, retracted nuclei (A3). Scale bar = 10 μm. Magnification = 400×.

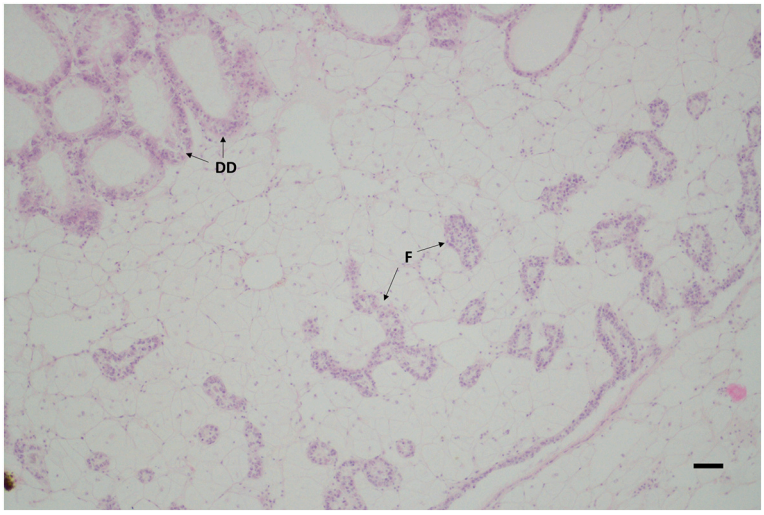

Fig. 5. Triploid *Crassostrea virginica* classified as inactive. Inactive triploids had little follicle development and no gametes. F: gonadal follicle; DD: digestive diverticula. Scale bar = 50 μm. Magnification = 100×.

clear criteria and the abnormal morphology of triploid gonads, there were an additional 17 individuals where it was unclear whether spawning occurred.

3.5. Classifying gonad development in triploids

The two major aspects for judging stages of sexual maturation in diploids – follicle development and gamete production – were asynchronous in triploids. For example, triploids with well-developed follicles sometimes lacked gametes, and sometimes rudimentary follicles were filled with spermatozoa. Without a clear connection between follicle and gamete development, triploids could not be accurately assigned to a stage of gonad development like that used for diploids.

Categories of gonad development for triploids were elicted from observations in this experiment. Triploids were classified based on the most abundant type of gonia present (α or β), the presence of spermatogenic cells or oocytes, and in some cases, the relative abundance of gametes.

3.5.1. Inactive triploids

In a few cases, triploids had little follicle development, few gonia, and no oocytes or spermatogenic cells and were considered inactive (Fig. 5).

3.5.2. Triploid males

Triploids with follicles containing spermatogenic cells (spermatocytes, spermatids, or spermatozoa) and mostly α gonia were considered males (Fig. 6). All triploid males had follicles containing a proliferation of primary spermatocytes. Follicles in triploid males usually contained a small number of spermatids and rarely contained spermatozoa. Spermatozoa were observed in 21 out of 196 males, and except for the one triploid that resembled a ripe diploid male, spermatozoa in triploid males were in much lower abundance than typical for diploids.

3.5.3. Triploid females, Oligo females, and Virilescent females

Triploids that contained mostly β gonia were classified as female, oligo female, or virilescent female. “Female” triploids contained one or more oocytes, on average, in each follicle (Fig. 7). In oligo (Greek, meaning few) females, none or a few oocytes were observed among all follicles (Fig. 8). Virilescent females were classified by the presence of spermatogenic cells within follicles lined with β gonia (Fig. 9). In virilescent females, it was common for some follicles to be empty and for some to contain small populations of spermatogenic cells. The name derives from descriptions by Han et al. (2010) of “spermatogenic-like cells” appearing in unproductive ovaries of triploid *Oncorhynchus mykiss*, which the authors referred to as “virilescent tendencies.” The lumina of follicles in virilescent females sometimes contained only spermatids or spermatozoa (Fig. 9), which was not observed in male triploids.

3.5.4. Hermaphrodites

Triploids were only considered hermaphrodites if substantial numbers of spermatogenic cells and oocytes were present simultaneously (Fig. 10). Hermaphrodites contained primarily β gonia, α gonia, or similar numbers of each.

3.6. Categories of triploids by site and time

Only triploid oysters sampled between May and August were classified based on gonad development (913 total). Four triploids could not be assigned to a category due to failure of histology. Of the 909 triploids that could be assigned to a category, 13 were inactive (~1%) and 5 were hermaphrodites (< 1%). The remaining 891 triploids were classified as male, female, oligo female or virilescent female (Fig. 11). The percentage of triploids assigned to these categories were similar at RR and ND throughout late spring (May 9–June 7) (Fig. 11). In early May, the percentage of males was 24% at ND and 33% at RR; in late May, 17% and 11%, respectively; in early June, 16% and 4%, respectively. Females made up a small, relatively unchanging percentage throughout late spring, ranging from 6% to 8% at ND and 5% to 11% at RR. Oligo females were most common at both sites in early May (ND: 53%; RR: 43%) and their frequency increased by late May (ND: 54%; RR: 58%) and increased again by early June (ND: 64%; RR: 71%). Virilescent females made up a similar percentage at ND and RR in early May (ND: 17%; RR: 10%), late May (ND: 21%, RR: 23%), and early June (ND: 11%, RR: 12%).

Differences among the percentage of triploids in categories were apparent between ND and RR in the summer (Fig. 11). In late June, there was a lower percentage of oligo females at ND (56%) than at RR (79%), as well as a higher percentage of males at ND (30%) than at RR (7%). The percentage of oligo females was even more dissimilar between the sites in July. Just 32% of triploids at ND were oligo females compared to 77% at RR. Also in July, females were more common at ND (35%) than at RR (15%), virilescent males were more common at ND (17%) than RR (3%), and males were more common at ND (13%) than RR (3%). In August, the percentages among categories at ND and RR were similar; however, females were now more common at RR (32%) than ND (18%) (Fig. 11).

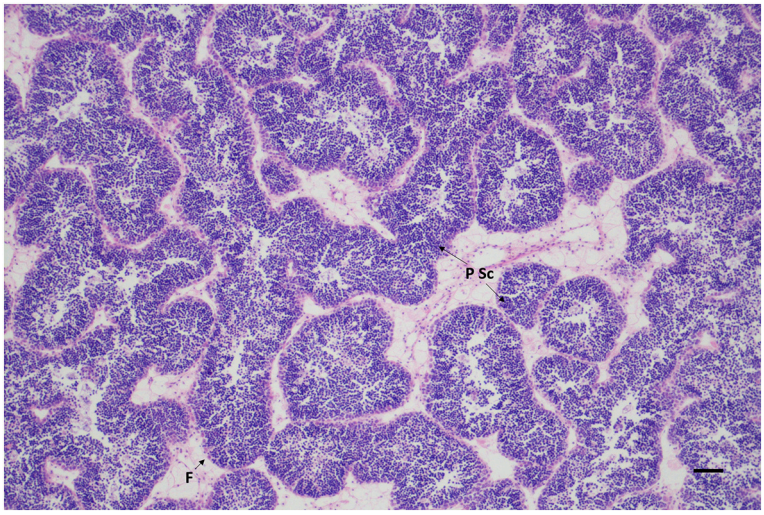

Fig. 6. Triploid *Crassostrea virginica* classified as male. Follicles are lined with α gonia and contain many primary spermatocytes. F: gonadal follicle; P Sc: primary spermatocyte. Scale bar = 50 μm. Magnification = 100×.

3.7. Categories of triploids by cross at ND, the site of triploid mortality

No major differences were observed among the four triploid crosses on May 11, the sampling just prior to the triploid mortality event at ND (Table 1). Among all crosses, females were rare (< 15%) and oligo females were most common.

3.8. Live vs. moribund triploids at ND

Some of the moribund triploids sampled during the mortality event at ND were processed histologically and classified based on gonad development (n = 30). The percentage of females was higher in moribund (23%) than live (8%) triploids, but no significant difference was found between the live and moribund triploids from Fisher's Exact Test (p = 0.2) (Table 2).

4. Discussion

4.1. Gametogenesis in triploid oysters

Gamete production in triploid *C. virginica* was greatly inhibited. Histological sections in males typically contained no spermatozoa, and sections from females usually contained only a few oocytes. These generalities are in agreement with previous observations in chemical *C. virginica* (Barber and Mann, 1991; Lee, 1988), and mated triploid *C. virginica* (Guévélou et al., 2019; Wadsworth et al., 2019). Similar findings have also been observed in chemical triploid *C. gigas* (Allen and Downing, 1990; Gardner et al., 1994; cf. Normand et al., 2008) and chemical triploid *S. commercialis* (Cox et al., 1996). Gamete production in mated *C. gigas* may be less impaired. From histological observations, Jouaux et al. (2010) reported that all triploid *C. gigas* examined had produced oocytes or spermatozoa, suggesting that among triploid oysters, mated triploid *C. gigas* may be especially fecund.

Triploid *C. virginica* can produce gametes; however, the possibility that their gametes would produce viable offspring, that is, their reproductive potential, is very low. Reproduction could theoretically occur from triploid × triploid or triploid × diploid mating. Based on fecundity and survival of progeny in hatchery settings, Guo and Allen (1994) estimated the reproductive potential from triploid × triploid crosses of *C. gigas* was 0.0008% of diploids and from diploid × triploid crosses was 0.0046% of diploids. Gong et al. (2004) estimated a higher reproductive potential from diploid × triploid crosses (0.1075%), owing partly to a much higher estimation of fecundity in triploid females of *C. gigas*. Although survival from triploid × triploid or diploid

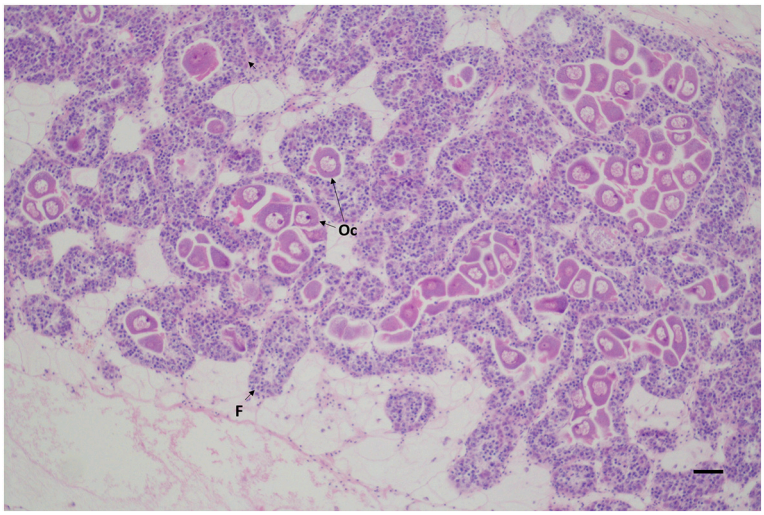

Fig. 7. Triploid *Crassostrea virginica* classified as female. Follicles are lined with β gonia and contain numerous oocytes. F: gonadal follicle; Oc: oocyte. Scale bar = 50 μm. Magnification = 100×.

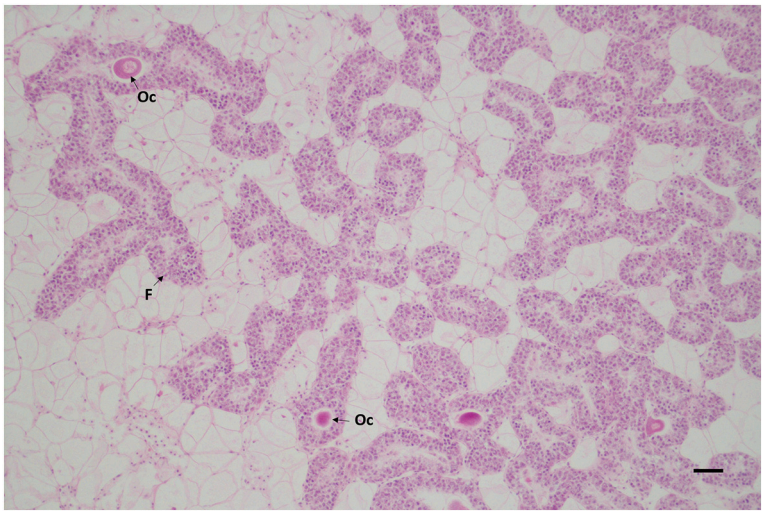

Fig. 8. Triploid *Crassostrea virginica* classified as oligo female. Follicles are lined with β gonia and contain few to no oocytes. F: gonadal follicle; Oc: oocyte. Scale bar = 50 μm. Magnification = 100×.

× triploid crosses has not been estimated in *C. virginica*, reproductive potential of triploid *C. virginica* may be even lower than that estimated in triploid *C. gigas* due to lower fecundity. Only 170 of 909 triploid *C. virginica* in this study (19%) were considered to have substantial numbers of observable gametes (males and virilescent females with spermatozoa, hermaphrodites, and females), while histological studies in triploid *C. gigas* found 25% (Jouaux et al., 2010) and 46% (Hermabessiere et al., 2016) of triploid *C. gigas* contained numerous gametes. Additionally, Peachey and Allen (2016) estimated the fecundity of triploid *C. virginica* females to be about 1.2% of diploid *C. virginica*, lower than both the 2% (Guo and Allen, 1994) and 13.4% (Gong et al., 2004) measured for estimating reproductive potential of triploid *C. gigas*.

In general, gonad development in (mated) triploid *C. virginica* deviated considerably from the typical development in diploids. In triploids, irregular gonia (β gonia) were common, which closely matched descriptions of "abnormal oogonia" (Allen and Downing, 1990) and "abnormal gonia" (Jouaux et al., 2010) in triploid *C. gigas*. Also, unlike the more uniform gonad development found among a cohort of diploid oysters, follicle and gamete development varied considerable from oyster to oyster in triploids. High variation in development in triploids was exacerbated due to a lack of synchrony between follicle development and gamete production. For example, some triploids had extensive follicle development and no gametes, whereas some had relatively undeveloped follicles filled with spermatozoa.

The lack of synchrony between follicle development and gamete production made it difficult to determine the stage of gametogenesis in triploids. Synchrony between follicle and gamete development is fundamental to the stages of development defined in diploid *C. virginica* (Kennedy and Battle, 1964; Loosanoff, 1942), and without it, gonads in triploid oysters did not correspond with development stages created for diploid *C. virginica*. Our method was to categorize gonads of triploid oysters without regard to a stage of development. We developed six categories for classifying gonads in triploid oysters, and the principal categorization was based on the gonia. Gonia were useful in distinguishing triploid oysters because regular gonia (α gonia) and abnormal gonia (β gonia) rarely coexisted in similar numbers. Additionally, the type of gonia largely dictated subsequent gamete development. The α gonia were reserved to males, and triploid females always contained β gonia. The close association between the type of gonia and sex lead us to hypothesize that α gonia are spermatogonia and β gonia are abnormal oogonia.

The morphology of α and β gonia provides some supporting evidence for the hypothesis. The α gonia in triploids were morphologically

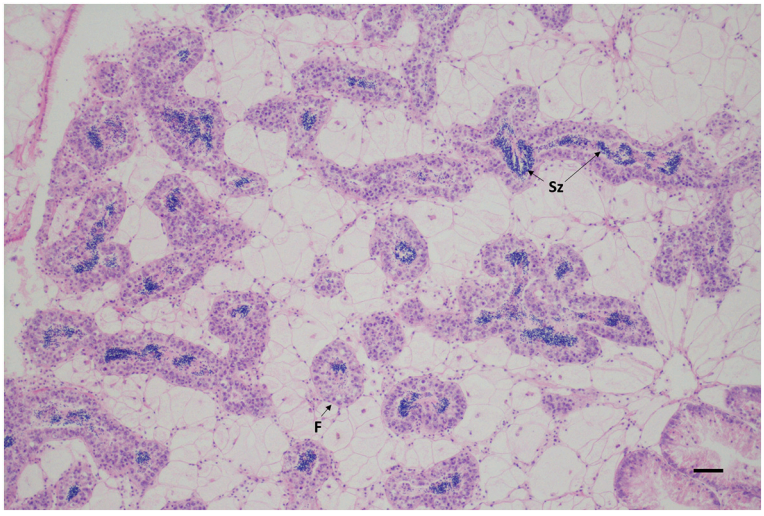

Fig. 9. Triploid *Crassostrea virginica* classified as virilescent female. Follicles are lined with β gonia and contain spermatozoa. F: gonadal follicle; Sz: spermatozoa. Scale bar = 50 μm. Magnification = 100×.

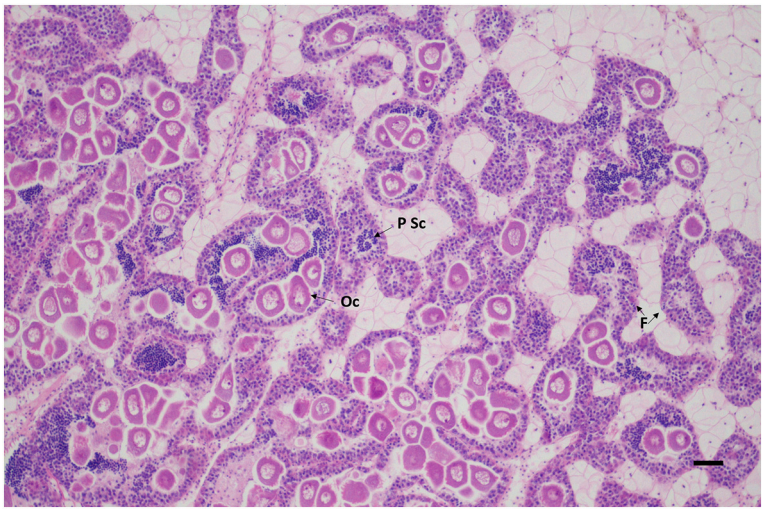

Fig. 10. Triploid *Crassostrea virginica* classified as hermaphrodite. Follicles contain many oocytes and primary spermatocytes. F: gonadal follicle; Oc: oocyte; P Sc: primary spermatocyte. Scale bar = 50 μm. Magnification = 100×.

identical to the spermatogonia in diploid males in this study, and the α gonia match descriptions of spermatogonia in diploid *C. virginica* by previous authors. Eckelbarger and Davis (1996a) observed spermatogonia in diploid *C. virginica* using electron microscopy and described them to have a single nucleolus and a spherical nucleus containing sparse heterochromatin, all of which correspond to the morphology of α gonia. For β gonia, their enlarged size may be evidence that oogenesis was initiated. Kennedy and Battle (1964) described oogonia in diploid *C. virginica* to have a larger nucleus (diameter: 5.6–6.0 μm) than spermatogonia (diameter: 3.2–4.1 μm). Eckelbarger and Davis (1996b) examined oogenesis in diploid *C. virginica* using electron microscopy and did not observe a distinct population of "mitotically dividing oogonia," but did measure "premeiotic" and "previtellogenic" oocytes of similar size to the "oogonia" described in Kennedy and Battle (1964). In our study, β gonia were generally larger than α gonia, and, similar to the oogonia described by Kennedy and Battle (1964), may have been in the process of enlarging and differentiating from oogonia to oocyte.

Spermatogonia developing normally (α gonia) and oogonia developing abnormally (β gonia) would corroborate previous findings in triploid oysters and several species of triploid fish. A common pattern in triploid oysters (Allen and Downing, 1990; Barber and Mann, 1991; Guévélou et al., 2019; Lee, 1988) and triploid fish (Benfey, 1999; Piferrer et al., 2009) is for the early, mitotic stages of spermatogenesis to appear to proceed normally and result in a proliferation of primary spermatocytes, but for meiotic daughter cells (secondary spermatocytes, spermatids, and spermatozoa) to be relatively absent. In contrast, oogenesis in triploids is affected at the earliest stages, inhibiting the production of normal, early stage primary oocytes and resulting in abnormalities in oogonia (in fish: Carrasco et al., 1998; Li et al., 2018; Piferrer et al., 1994; Solar et al., 1984; in oysters: Allen and Downing, 1990).

Jouaux et al. (2010) had a different assessment of abnormal gonia in triploid *C. gigas*. Jouaux et al. (2010) found abnormal gonia to be common in male and female triploid *C. gigas* and considered the abnormal gonia to be arrested in prophase, referring to them as "locking events." The authors examined triploid oysters throughout the course of gametogenesis, and at each stage, some triploids (both male and female) had locking events and disturbed gametogenesis, whereas the rest had no locking events and had a diploid-like gonad. Jouaux et al. (2010) thus suggested that there were two types of "gametogenic paths," a path with locking events, disturbed gametogenesis, and production of few gametes (β), and a path without locking events, a gonad development very similar to a diploid, and production of many gametes (α).

Gonad development in triploid *C. virginica* bears little resemblance to that described by Jouaux et al. (2010) for triploid *C. gigas*. In triploid *C. virginica*, gonad development is always abnormal, and a strong relationship between normal gonia and fecundity is absent. Thus, the same dichotomous system described by Jouaux et al. (2010) seems inapt for triploid *C. virginica*.

Based on our observations, we hypothesize major pathways for gonad development in triploid *C. virginica* (Fig. 12). The proposed pathways only involve the categories of gonad development that were commonly observed in this study — males, females, oligo females, and virilescent females — and only consider development within a single reproductive cycle. The pathways therefore do not address any changes that could occur post-spawning.

The first bifurcation in gonad development of triploid *C. virginica* is the state of the gonia. We hypothesize that if gonia are actuated as spermatogonia (α gonia), primary spermatocytes will proliferate, but few to no secondary spermatocytes, spermatids, or spermatozoa will be produced. If gonia proceed on a female pathway, gonial proliferation and differentiation are abnormal, indicated by β gonia. The initiation in either the male or female pathway is likely determined early through a combination of environmental and genetic mechanisms unclear at this time (e.g., Guo et al., 1998; Hedrick and Hedgecock, 2010; Zhang et al., 2014), especially for triploids (Dheilly et al., 2014).

Those follicles on the female path, we suggest, have several possible outcomes. Most of the time, triploids on the female path produce few oocytes and are regarded as "oligo females," while a much smaller proportion produce a substantial number of observable oocytes and are called "females." The gonad structure among the three types of females — oligo females, females, and "virilescent females" — are highly similar, with the sole distinction being the contents of the follicles. Thus, we suggest that oligo females may 1) remain oligo females 2) produce oocytes and become more fecund and manifest as "females," representing delayed oocyte production like that previously reported in *C. gigas* (Allen and Downing, 1990), or 3) produce spermatogenic cells and become virilescent females. The decrease in oligo females and coincident increase in females and virilescent females between late June and early July at ND provides evidence that these transitions can occur (Fig. 11).

Virilescent females, defined in this study as triploids that start with aberrant oogenesis and then produce spermatogenic cells, have been observed in triploid fish. Spermatogenic cells appearing in underdeveloped ovaries of triploids has been observed in rainbow trout (*O. mykiss*) (Carrasco et al., 1998; Han et al., 2010) and hybrid groupers (*Epinephelus spp.*) (Li et al., 2018). Carrasco et al. (1998) hypothesized

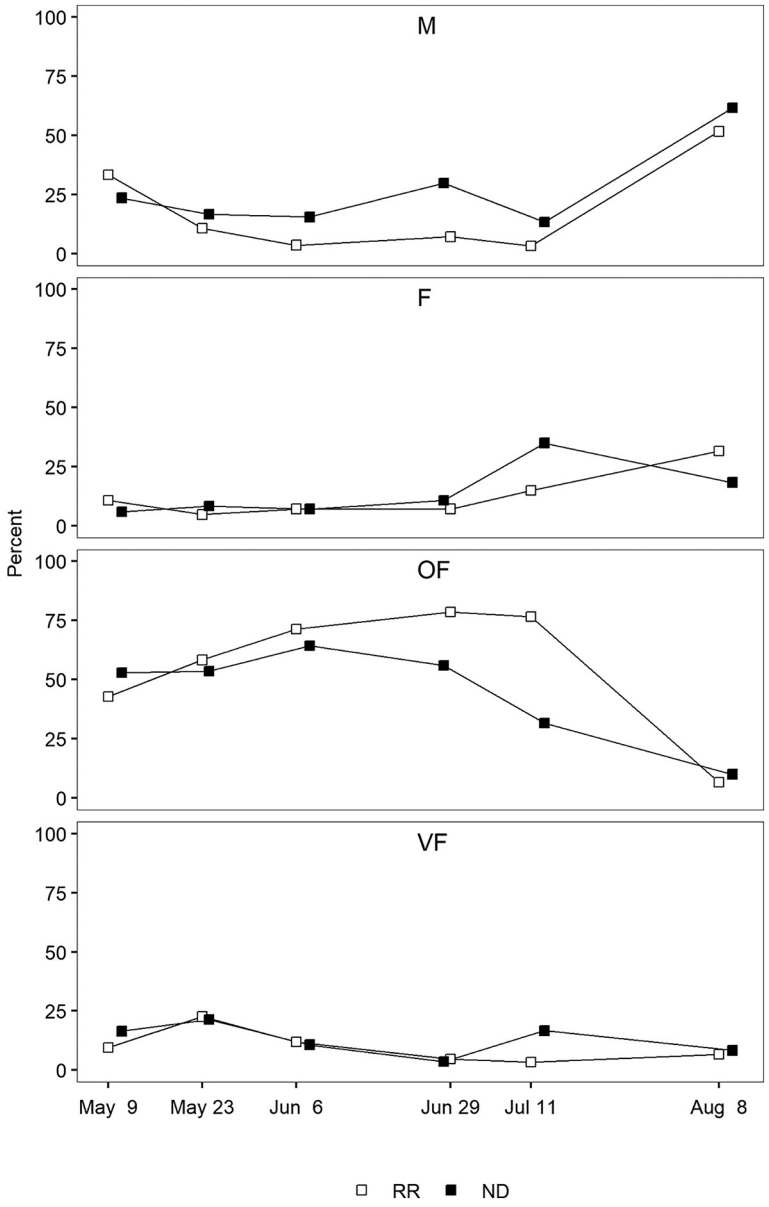

Fig. 11. Percentage of triploid *Crassostrea virginica* individuals of each category of gonad development sampled from Rappahannock River (white) and Nandua Creek (black) in 2016. Inactive and hermaphrodites were rare between May and August (≤ 1% of total) and are not included in the fig. ND: Nandua Creek; RR: Rapphannock River; M: Male; F: Female; OF: Oligo female; VF: Virilescent female.

Table 1
Percentage of individuals classified in each category of gonad development for four crosses of triploid *Crassostrea virginica* sampled from Nandua Creek on May 11 just prior to the triploid mortality event (for LLV $n = 20$; for VVV and VVM $n = 21$; for LLM $n = 22$).

	I	M	F	OF	VF
VVV	0%	14%	5%	67%	14%
VVM	0%	14%	5%	57%	24%
LLV	5%	33%	14%	43%	5%
LLM	0%	32%	0%	45%	23%

The triploid crosses (VVV, VVM, LLV, and LLM) are named based on the origin of their tetraploid sires (Virginia, VV or Louisiana, LL) and diploid dams (Virginia, V, or Maine, M). I: Inactive; M: Male; F: Female; OF: Oligo female; VF: Virilescent female.

Table 2
Percentage of individuals classified in each category of gonad development for moribund ($n = 30$) and live ($n = 84$) triploid *Crassostrea virginica* sampled during a triploid mortality event in Nandua Creek on May 24, 2016. M: Male; F: Female; OF: Oligo female; VF: Virilescent female.

	M	F	OF	VF
Moribund	13%	23%	50%	13%
Live	17%	8%	54%	21%

that the “male-differentiating areas” were a result of abnormal persistence of clustering oogonia, which interfered with somatic-germ cell interactions necessary for ovarian development. Without normal somatic-germ cell interactions, somatic cells that would otherwise aid in

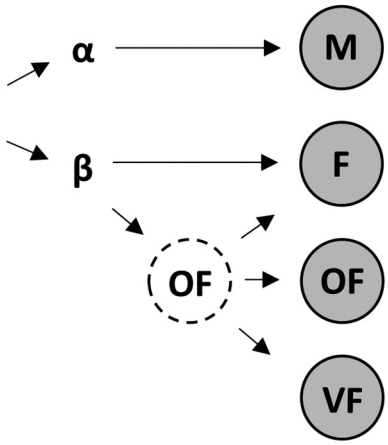

Fig. 12. Proposed pathways for gonad development in triploid *Crassostrea virginica* within a single reproductive cycle. Initially, triploids either undergo spermatogenesis and produce α gonia (α) or undergo oogenesis and produce β gonia (β). Triploids with initial spermatogenesis become triploid males (M). Oogenesis may lead to a substantial number of observable oocytes resulting in triploid females (F) or may lead to triploids with few oocytes, or oligo females (OF). Oligo females may produce more oocytes and become females, produce spermatogenic cells and become virilescent females (VF), or remain oligo females.

oogenesis differentiate into cells supporting spermatogenesis, resulting in production of spermatogenic cells and what Han et al. (2010) referred to as "virilescent tendencies" (Carrasco et al., 1998). Important cell interactions may also be inhibited by the persistence of β gonia in triploid *C. virginica*, which were regularly observed lining the gonadal follicles in several layers.

A connection between failed oocyte production and spermatogenesis, which may be represented by virilescent females, has already been proposed in triploid oysters by Dheilly et al. (2014). In triploid *C. gigas*, Dheilly et al. (2014) found "stage III β females," females at the end of the reproductive cycle with few oocytes, upregulating genes associated with spermatogenesis. The finding led Dheilly et al. (2014) to propose that such "disruption of sex differentiation mechanisms" may be responsible for failed gamete production in triploids. Interestingly, the virilescent females in our study, which may represent unproductive females upregulating genes associated with spermatogenesis, were especially successful in producing spermatozoa. Spermatozoa were much more common in virilescent females than in triploid males, suggesting that in *C. virginica*, such "disrupted sex differentiation mechanisms" may promote fecundity, at least in terms of successful spermatozoa production. A possibly important factor related to spermatogenesis in virilescent females and triploid males is the surrounding cellular environment. Triploid males always had follicles filled with large numbers of primary spermatocytes, while virilescent females often exhibited many empty follicles or follicles with relatively few primary spermatocytes.

Our proposed pathways for gonad development do not fully explain our time-series data because triploids spawned during the experiment. Between July and early August, the time which spawning primarily took place in triploids, the percentage of males increased. Considering the starkly different architecture of the gonad between oligo females and males, it is unlikely that oligo females became males. Interestingly, however, the increase in percentage of males in the triploids coincided with an increase in the percentage of males in the diploids. Sex change between seasons is known to occur in *C. virginica* (Needler, 1932); however, it has not been documented between spawns in the same year. Conclusive results on changes in sex and pathways of development in triploid *C. virginica* may only be possible with repeated measures on individual oysters (e.g. Needler, 1932).

4.2. Gametogenesis and triploid mortality

A link between gametogenesis and triploid mortality was investigated with oysters from the site where a triploid mortality event occurred, Nandua Creek (ND), and those where it did not occur, Rapphannock River (RR). The sites are on different sides (east-west) of the lower Chesapeake Bay and have different salinity profiles (ND: 16–20 ppt; RR: 11–15 ppt), yet both temperature and the growth rate of the experimental oysters were similar at ND and RR during the field trial (Matt et al., 2020).

Diploids at ND were gravid weeks earlier than diploids at RR and thus had an earlier onset of gametogenesis or more rapid gonad development than diploids at RR. Given mean daily temperatures were similar between the sites (Matt et al., 2020), differences in gametogenesis may have been related to food supply, which can affect the extent (Delaporte et al., 2006; Jouaux et al., 2013; Samain and McCombie, 2008) and rate of gametogenesis (Dutertre et al., 2009; Liu et al., 2010). In contrast, the gonads in triploids at ND and RR were similar throughout the experiment. Except for the mid-summer sampling when fecund females were more common at ND, the percentage of triploids in each category of gonad development at ND and RR was similar from May to August.

Susceptibility to triploid mortality may not depend on the morphology of gonad development. Although we could only examine 30 moribund oysters sampled during the triploid mortality event, gonad morphology of moribund and live triploids was similar. Our findings confirm those by Guévélou et al. (2019) and Wadsworth et al. (2019), that gonad development in triploids did not explain differential mortality.

Triploid mortality, like summer mortality in *C. gigas*, may be due to a "physiological disorder" related to reproduction (Koganezawa, 1975). Measuring the variance in what is essentially reproductive sterility does not quantify the underlying physiological processes in triploid *C. virginica*, where arrested gonad development is undoubtedly disrupting many other pathways. Better insight into the cause of triploid mortality could come from examining components of metabolism during gametogenesis, such as changes in biochemical energy reserves. In bivalves, the concentration of biochemicals such as glycogen, lipid, and protein, often exhibit a seasonal cycle influenced by gametogenesis (Deslous-Paoli and Héral, 1988; Gabbott and Stephenson, 1974; Mann, 1979; Masumoto et al., 1934).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix

Table A.1
Descriptions of stages of gametogenic development used for diploid *Crassostrea virginica*.

Stage	Description
Inactive (I)	Dormant, very few or no follicles present ($\leq$ 5% follicle coverage).
Very Early Active (VEA)	Few small follicles devoid of lumina. Only gonia (no immature oocytes or spermatocytes) present. Some follicles may be branching (10–30% follicle coverage).
Early Active (EA)	Follicles have lumina and are largely branched. Immature oocytes proliferating in females, proliferation of primary and secondary spermatocytes in males (10–40% follicle coverage).
Active (A)	Follicles branched and conjoined to form canals. Mature oocytes outnumber immature oocytes. Spermatids and spermatozoa present in males. Some connective tissue remains (50–70% follicle coverage).
Late Active (LA)	Pronounced follicle canals. Large oocytes disconnected from follicle wall in females, spermatozoa most common contents of follicles in males. Little connective tissue remains (75–90% follicle coverage).
Ripe (R)	Follicles distend from mantle to digestive tissue, either filled with oocytes or spermatozoa. Nearly no connective tissue remains ($\geq$ 80% follicle coverage).
Spawning (S)	Slightly shrunken follicles and irregular arrangement of oocytes in females. In males, irregular arrangement of spermatozoa. Follicles in males may be partially empty. Gametes present in ducts and appear to be entering ducts from neighboring interconnected follicles. Still little connective tissue present (60–90% follicle coverage).
Advanced Spawning (AS)	Continuation of follicle contraction in females. Follicles more empty in males. More connective tissue present in females and males (30–70% follicle coverage).
Spawned Out (SO)	Collapsed follicles and disorganization of the gonad. Hemocytes may be present and connective tissue makes up majority of the gonad area ($<$ 50% follicle coverage).

Stages are similar to that used by Kennedy and Krantz (1982) for *C. virginica*, Allen et al. (1986) for *Mya arenaria*, and Allen and Downing (1990) for *C. gigas*. Stages are distinguished based on follicle contents as well as percent follicle coverage, defined as the percent of the incipient gonad area occupied by gonadal follicles. The incipient gonad area was defined as the area between the digestive tissue and mantle.

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