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STERILE TRIPLOID *CRASSOSTREA VIRGINICA* (GMELIN, 1791) GROW FASTER THAN DIPLOIDS BUT ARE EQUALLY SUSCEPTIBLE TO *PERKINSUS MARINUS*¹

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ABSTRACT Growth, tolerance of *Perkinsus marinus*, and gametogenesis of diploid and triploid Eastern oysters, *Crassostrea virginica* (Gmelin, 1791) were compared in the York River, Virginia between June 1989 and November 1990. Triploid oysters had significantly greater mean shell height ($P \leq 0.02$) and whole weight ($P \leq 0.005$) than diploid oysters throughout the study period. In November 1990, triploids had significantly greater mean dry tissue weight ($P \leq 0.006$) than diploids. On average, triploid oysters reached commercial size (63.5 mm) 5 months before diploid oysters. Diploid and triploid groups became similarly infected with *P. marinus* during summer 1990. Prevalences reached 96% with moderate and heavy intensities in both groups in October 1990. Gonadal development in triploids was significantly reduced ($P \leq 0.02$) compared to development in diploids. While diploid oysters completed a normal gametogenic cycle, gametogenesis in triploid male oysters was arrested prior to gamete maturation in all but one individual, and in triploid females only a few isolated oocytes were produced. Potential commercial utilization of triploid *C. virginica* will most likely take advantage of superior growth rates compared to diploids, as disease tolerance was not improved.

KEY WORDS: oysters, triploid, growth, gametogenesis, disease, *Crassostrea virginica*, *Perkinsus marinus*

INTRODUCTION

Triploid oysters (having three sets of chromosomes) represent an increasing proportion of hatchery oyster (*Crassostrea gigas* Thunberg) production in the U.S. Pacific Northwest (Oregon and Washington), which now accounts for approximately 37% of total U.S. production (U.S. National Marine Fisheries Service, 1990 Landings for the United States). The commercial value of triploid oysters results from the physiological alteration that occurs as the result of having an extra set of chromosomes. Most notably, triploid *C. gigas* exhibit retarded gonadal development compared to diploid (normal) oysters (Allen and Downing 1986, 1990). Thus being relieved of the metabolic requirements of gametogenesis, triploid oysters exhibit greater somatic growth and a more consistent glycogen content than diploid oysters, making them a better market product throughout the year (Allen and Downing 1986, Davis 1989). Triploid *C. gigas* are also less susceptible to "summer mortality," which has been related to stress induced by excessive gonadal maturation (Perdue et al. 1981, Beattie et al. 1988).

As reviewed by Haskin and Andrews (1988) and Andrews (1988), native stocks of *Crassostrea virginica* Gmelin along the east coast of the United States have been severely depleted by two oyster pathogens, *Haplosporidium nelsoni* and *Perkinsus marinus*. At sublethal levels, the diseases caused by these pathogens inhibit growth, reduce fecundity, and lower condition and glycogen content (Menzel and Hopkins 1955, Newell 1985, Barber et al. 1988a, 1988b, Crosby and Roberts 1990, Paynter and Burreson 1991). Although resistance to mortality caused by *H. nelsoni* has been achieved through selective breeding (Ford and Haskin 1987), no increase in tolerance to *P. marinus* has been effected to date. Thus the impact of *P. marinus* continues unabated.

Evaluation of the performance of triploid *C. virginica* with respect to growth, disease tolerance, and gametogenesis is limited. Compared to their diploid counterparts, triploid *C. virginica* have

been reported to grow faster (Stanley et al. 1984), exhibit inhibited gametogenesis (Lee 1988), and have similar susceptibility to *P. marinus* in dosed flumes (Meyers et al. 1991). This study provides the first coordinated examination of growth, disease tolerance, and gametogenesis of triploid *C. virginica* in the field, so a preliminary evaluation of the potential value of triploid oysters to the aquaculture industry of the eastern U.S. can be made.

MATERIALS AND METHODS

Broodstock oysters obtained from Mobjack Bay, Virginia, were conditioned in the Virginia Institute of Marine Science (VIMS) hatchery in early 1989 at 20–24°C with a diet consisting of *Isochrysis galbana* (Tahitian), *Thalassiosira pseudonana* (3H), *T. weissflogii*, and *Chaetoceros calcitrans*. Spawning was induced by elevating the temperature to 28–30°C and adding a sperm suspension, if necessary. Triploid larvae were produced from oysters (3♀ and 2♂) spawned on 23 March, 1989, and diploid larvae were produced from oysters (6♀ and 8♂) spawned on 5 April, 1989. Triploidy was induced by adding (15 min after fertilization) 1.0 mg cytochalasin B (CB) in 1.0 ml DMSO to a 2 l beaker containing embryos in 1 l of seawater. Treatment with CB lasted 15 min and was followed by a 15 min DMSO (1.0 ml/l) exposure (Downing and Allen 1987, Allen et al. 1989).

Larvae were reared in 400 gal conical tanks. Water was changed every 1–2 days and food (*I. galbana*, *T. pseudonana*, *T. weissflogii*, and *C. calcitrans*) was added 1–2 times daily. Eyed larvae were set onto "minicultch" (ground oyster shell) and placed into upwellers receiving raw water from the York River, Virginia. Oysters were removed from the upwellers in July and placed into trays that were hung off a pier at the VIMS campus (York River, Virginia). Twenty-five individuals from each group were assayed for ploidy by Dr. S. Allen, Rutgers University, using flow cytometry (Chaiton and Allen 1985). The CB-treated group contained 96% triploids, while the untreated group contained 0% triploids.

Regular determinations of shell height (maximum dimension from hinge to opposite margin) and whole (live) weight, began in June and August 1989, respectively, and were continued until

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November 1990. The number of individuals measured was 30 per group prior to November 1989 and 50 per group from November 1989 onward. Additionally, in November 1990, 60 oysters from each group were shucked for determination of dry tissue weight (80°C).

Beginning in August 1989, 25 individuals from each group were sacrificed periodically for determination of prevalence and intensity of *Perkinsus marinus*, using the fluid thioglycollate method (Ray 1952). Prevalence was the percentage of oysters found to have infections, and the intensity of infection in each oyster was rated as light (L), moderate (M), or heavy (H) (Ray 1954).

Gametogenesis was monitored from April through October 1990. Twenty individuals from each group were sacrificed and fixed in Davidson's AFA. A standard transverse (anterior) section of the visceral mass taken beginning at the level of the intersection of the labial palps and gills (and including gill, mantle, stomach, intestine, and digestive diverticula) was then dehydrated, cleared, and embedded in Paraplast. Six- μ m sections were mounted on slides and stained with Harris' Hematoxylin and Eosin Y. To quantify gonad development, a gonadal area index (GAI) was determined from the histological section of individuals found to be sexually differentiated, as the ratio of [gonadal area/area of entire visceral mass] \times 100. GAI represents the proportion of total cross sectional area that is comprised of gonadal tissue and is indicative of gonadal development (gametogenesis) and spawning (Barber et al. 1988a, 1991).

Comparisons of mean shell height, whole weight, dry tissue weight (November 1990 only), and GAI (after arcsin transformation) between diploid and triploid groups were made for each sampling date using a t-test.

RESULTS

Growth

Growth of all oysters (measured both as shell height and whole weight) occurred primarily in fall (September–December) 1989 and 1990 and spring (April–July) 1990 but slowed considerably during winter (December–April) 1989–90 and summer (July–October) 1990 (Figures 1 and 2).

Mean shell height of diploid oysters increased from 10.9 mm in June 1989 to 64.6 mm in November 1990, while mean shell height

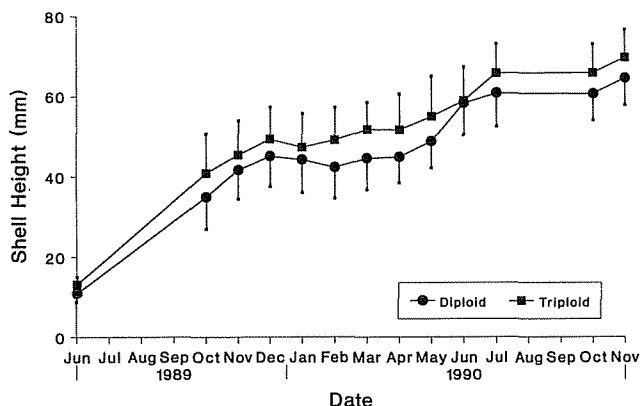


Figure 1. Mean shell height (mm) of diploid and triploid oysters, *C. virginica*, grown in the York River, Virginia, between June 1989 and November 1990. Error bars represent 1 SD.

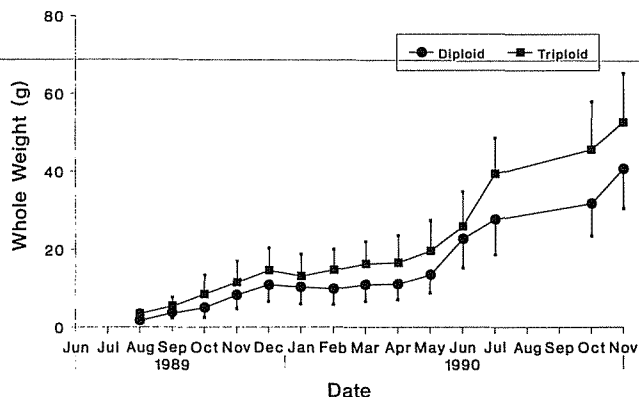


Figure 2. Mean whole weight (g) of diploid and triploid oysters, *C. virginica*, grown in the York River, Virginia, between August 1989 and November 1990. Error bars represent 1 SD.

of triploid oysters increased from 13.1 mm to 69.7 mm over the same time interval (Figure 1). Means of the triploid group were significantly greater ($P \leq 0.02$) than means of the diploid group on all sampling dates except January and June 1990. From October 1989 through November 1990, triploid oysters maintained an average 5 mm shell height differential over diploid oysters. A mean shell height of 63.5 mm (considered commercial size) was attained by triploid oysters in July 1990 and by diploid oysters in November 1990 (Figure 1). In July 1990, 66% of the triploid group exceeded 63.5 mm in shell height compared to 38% of the diploid group.

Between August 1989 and November 1990, mean whole weight of diploid oysters increased from 1.6 g to 40.6 g, and mean whole weight of triploid oysters increased from 3.4 g to 52.6 g (Figure 2). Means of the triploid group were significantly greater ($P \leq 0.005$) than means of the diploid group at all sampling dates except June 1990. Over the course of the study, the differential in whole weight between triploid and diploid oysters increased. In August 1989, the difference between mean whole weight of diploid and triploid oysters was 1.8 g, but by October 1990 this difference had increased to 13.8 g (Figure 2).

In November 1990, mean dry tissue weight of triploid oysters was 1.1 g compared to 0.9 g for diploid oysters. These means were significantly different ($P \leq 0.006$).

Disease

P. marinus was not detected in diploid or triploid oysters (prevalence = 0%) in either August or September 1989, shortly after being placed into the York River, VA (Table 1). In October and again in November 1989, 1–2 individuals in each group had extremely light infections (1–2 *P. marinus* cells). In April 1990, prevalence was reduced to 0%. In July 1990, prevalence of *P. marinus* was 56% in the diploid group and 44% in the triploid group. Most infections were light, but there were also 3 moderate infections and 1 heavy infection in each group. By October 1990, prevalence had increased to 96% in both groups, with a concomitant increase in both moderate and heavy infections. Although no accurate counts were obtained, mortalities were observed in both diploid and triploid groups beginning in July 1990.

Gametogenesis

Mean GAI of both diploid and triploid oysters was 0% in April 1990 (Figure 3). Beginning in May, mean GAI of diploid oysters

TABLE 1.

Prevalence (%) and intensity (L = light; M = moderate; H = heavy) of *P. marinus* infections in diploid and triploid oysters, *C. virginica* from August 1989 to October 1990; n = 25.

Date	Group	Prevalence (%)	Intensity L-M-H
14 August 1989	Diploid	0	0-0-0
	Triploid	0	0-0-0
13 September 1989	Diploid	0	0-0-0
	Triploid	0	0-0-0
16 October 1989	Diploid	4	1-0-0 ¹
	Triploid	8	2-0-0 ¹
13 November 1989	Diploid	4	1-0-0 ¹
	Triploid	4	1-0-0 ¹
16 April 1990	Diploid	0	0-0-0
	Triploid	0	0-0-0
31 July 1990	Diploid	56	10-3-1
	Triploid	44	7-3-1
11 October 1990	Diploid	96	15-5-5
	Triploid	96	16-2-6

¹ These infections were based on the detection of only 1 or 2 *P. marinus* cells in the thioglycollate culture.

increased to values of 18.3% in June, 17.8% in July, and 16.8% in August. Mean GAI for the diploid group decreased to 10.5% in October. Mean GAI of triploid oysters never exceeded 8%. Mean GAI values for diploid and triploid groups were significantly different ($P \leq 0.02$) for all sampling dates after April 1990 (Figure 3), indicating that triploid oysters had considerably lower gonadal production than diploid oysters.

Diploid oysters were observed to undergo a typical gametogenic cycle. All individuals were undifferentiated in April (Table 2). By May gametogenesis was initiated, as 8 of 20 individuals were differentiated. In June, July, and August, 57 of 60 individuals were differentiated and contained oocytes and spermatocytes, as well as mature gametes (Figures 4A, 4B). In the October sample, it was obvious that spawning had occurred in 17 of the 20 individuals examined, as follicles were reduced in size and at least partially devoid of mature gametes. In a few cases, spawning and resorption were so complete that sex could no longer be determined (Table 2).

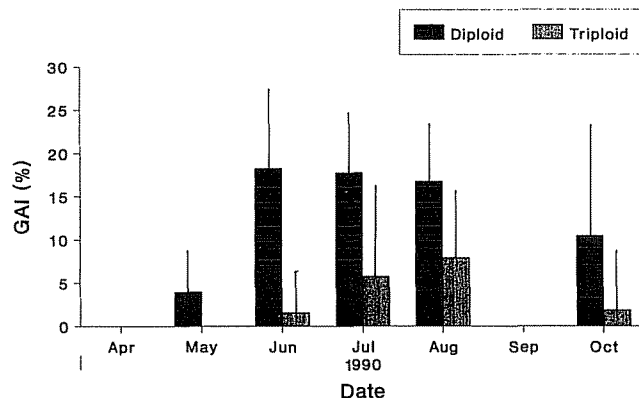


Figure 3. Mean gonadal area index (GAI, %) of diploid and triploid oysters, *C. virginica*, grown in the York River, Virginia, between May and October 1990. Error bars represent 1 SD.

TABLE 2.

Number of undifferentiated (U), male (M), and female (F) diploid and triploid oysters, *C. virginica*, from April–October 1990 and notes on gametogenesis; n = 20.

Date	Diploid Group U-M-F	Triploid Group U-M-F
16 April	20-0-0	20-0-0
21 May	12-7-1	20-0-0
20 June	2-11-7 (sperm; ova)	14-1-5 (1 had sperm) ^{1,2}
24 July	0-12-8 (sperm; ova)	7-6-7 (1 had sperm) ²
23 August	1-15-4 (sperm; ova)	4-13-3 (1 had sperm; 1 had ova) ²
11 October	6-9-5 (17 spawned)	16-3-1 (1 had ova; ² 0 spawned)

¹ This individual was a hermaphrodite.

² This individual was later identified as a diploid.

Triploid oysters did not undergo a normal gametogenic cycle. In both April and May, all individuals were undifferentiated (Table 2). In June, July, and August, the number of differentiated individuals increased from 6 to 13 to 16, respectively. Females

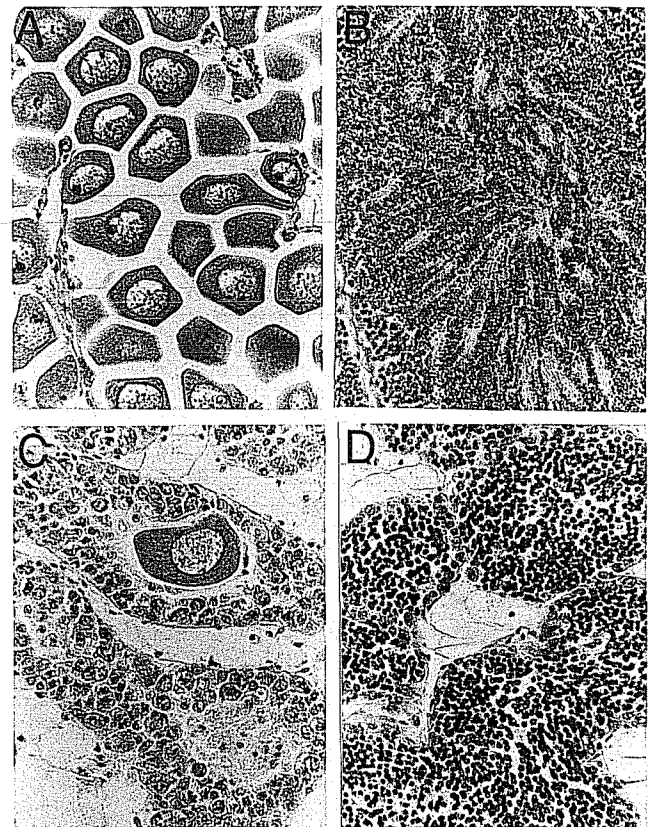


Figure 4. Gonadal sections of: (A) Mature diploid female with oocytes. (B) Mature diploid male with spermatocytes and spermatozoa. (C) Typical triploid female with single, isolated oocyte. (D) Typical triploid male with spermatocytes but no spermatozoa. Magnification of all plates is 400 \times .

were distinguished by the presence of very few oocytes, most often isolated in separate follicles containing no other developing oocytes (Figure 4C), and males typically contained spermatocytes but no spermatozoa (Figure 4D). In October, the number of undifferentiated individuals increased to 16 (Table 2). There was no evidence of spawning (follicles partially devoid of gametes or gametes in gonoducts) in any of the triploid oysters. Instead, infiltration of follicles by phagocytes was evident, indicating resorption of gametes.

Of the 120 oysters examined from the 96% triploid group, mature gonads were found in only five individuals, one of which was a hermaphrodite (Table 2). Samples of adductor muscle tissue from these individuals were subsequently assayed for ploidy (S. K. Allen, Rutgers University), and 4 of the five were diploid, the exception being a male from the 23 August sample.

DISCUSSION

Growth

Triploid oysters were significantly larger than diploid oysters, both in terms of shell height and whole weight, throughout the period of this study, which ended when oysters were about 1.5 yrs of age. The 2 week age difference between diploid and triploid groups, although potentially important initially, was not considered a factor after the age of 1 yr. A difference in shell height of about 5 mm was maintained between triploids and diploids throughout most of the study period. In the case of whole weight, which includes both shell and tissue components, the difference between triploids and diploids appeared to increase over time, suggesting that tissue production was greater in triploids. This was verified by the significantly greater dry tissue weight found in triploid oysters than in diploid oysters in November 1990.

Growth rate was generally greater during fall and spring than during winter and summer. Oyster growth is generally reduced during winter as the result of lower temperatures and decreased clearance rates. The decrease in growth rate during summer 1990 occurred in conjunction with infection by the oyster pathogen *P. marinus*, which has been shown to negatively affect oyster (shell) growth (Menzel and Hopkins 1955, Paynter and Bureson 1991). Gametogenesis also occurs in summer, and is known to retard shell growth in oysters (Mann 1979, Allen and Downing 1986). Because both diploid and triploid groups experienced reduced growth in summer 1990, disease, rather than differential gonadal development, is the most likely cause.

In general, because triploid oysters invest less energy in the production of gametes, more energy is available for somatic growth. Allen and Downing (1986) found that triploid *C. gigas* continue to grow and use less of their stored glycogen than diploids during the normal gametogenic period, when growth in diploids ceases. Growth differences between diploid and triploid *C. gigas* may depend upon environmental conditions and resulting glycogen utilization patterns (Davis 1988, 1989). Triploid scallops, *Argopecten irradians* and *Chlamys nobilis*, displayed greater somatic growth compared to diploid scallops as a result of retarded gonadal development and reduced glycogen utilization (Tabarini 1984, Komaru and Wada 1989). Superior growth of triploid organisms may also be associated with the increased heterozygosity resulting from the extra set of chromosomes. Stanley et al. (1984) reported that triploid *C. virginica* grew faster and were more heterozygous than diploid siblings only if triploidy were accomplished by blocking meiosis I. Triploid *Mya arenaria* were also

found to be nearly twice as heterozygous as their diploid siblings (Mason et al. 1988).

Disease

The results of this study indicate that triploid *C. virginica* are no more tolerant of *P. marinus* than diploid oysters. Because oysters were placed in the field late in 1989, infections incurred that year were rare and extremely light. These infections were apparently lost (or became subclinical) over winter, as *P. marinus* was undetected in April 1990. By July, however, about 50% of both diploid and triploid groups were infected, and this increased to 96% by October, with similar intensities and mortalities in both groups. Thus triploid *C. virginica* become infected with *P. marinus* in nature to the same extent as diploids in spite of their presumed energetic advantage. Similar results were reported for an experiment in which diploid and triploid *C. virginica* were dosed with *P. marinus* cells in a flume (Meyers et al. 1991). Tolerance to the effects of *P. marinus* must therefore be more specific than and unrelated to greater glycogen content associated with the lack of reproductive effort.

Although mortality comparisons between diploid and triploid groups were not made in this study, the similarity in susceptibility *P. marinus* suggests that mortalities would also be similar. This was confirmed by Meyers et al. (1991) who reported that cumulative mortality of *C. virginica* 150 days after being dosed with *P. marinus* was 100% and 98% for diploid and triploid groups, respectively.

The effect of *P. marinus* on oyster energy metabolism has not been examined to date, but it is recognized that *H. nelsoni* infection negatively affects oyster filtration rate, glycogen content, and condition (Newell 1985, Barber et al. 1988a, 1988b). Furthermore, oysters selected for tolerance of *H. nelsoni* exhibit greater filtration rates than susceptible oysters during periods of greatest parasite activity (Barber et al. 1991). Considering the inverse relationship between glycogen content and *H. nelsoni* infection intensity and the greater glycogen content of triploid oysters, an examination of the relationship between ploidy and infection of *C. virginica* by *H. nelsoni* would be a useful comparison to the findings presented here.

Gametogenesis

As indicated by the significantly lower GAI values, triploid *C. virginica* exhibited considerably reduced gonadal development compared to diploids. While diploid oysters underwent a normal gametogenic cycle their second full summer (1990) and subsequently spawned mature gametes, more triploid oysters failed to differentiate, and those that were differentiated produced only a few isolated oocytes or ova (females) and spermatocytes (males, only one having spermatozoa). Based on histological observation, these immature gametes were resorbed, rather than spawned. Thus the little gametogenic material that was produced by triploids did not develop into significant numbers of gametes and was not spawned. These observations are consistent with those previously reported for *C. virginica* by Lee (1988).

Gametogenesis in triploid bivalves is either uninitiated because of the inability of homologous chromosomes to synapse in meiosis or arrested because of multivalent formation and aberrant segregation (Allen et al. 1986). In the cases of *C. virginica*, *A. irradians*, *Mya arenaria*, and *C. nobilis*, gametogenesis is initiated, but rarely completed (Tabarini 1984, Allen et al. 1986, Lee 1988, Komaru and Wada 1989). We found only one confirmed triploid

individual with spermatazoa, and few, if any, with advanced oocytes. Thus triploid *C. virginica* may rarely produce mature gametes. A population of triploid *C. virginica*, however, would be effectively sterile if mature gametes are produced only by males or if gametes, once produced by either sex, are not spawned.

In contrast to *C. virginica*, triploid *C. gigas* produced both spermatazoa and ova, although in smaller quantities than diploid siblings (Allen and Downing 1986, 1990). Although the mechanisms involved are not fully understood, the extent of gametogenesis in triploid *C. gigas* appears to be greater than in *C. virginica*. As noted by Allen and Downing (1990), spawning of both mature and immature gametes occurs in triploid *C. gigas*, and normal embryos are produced. The ability of triploid *C. gigas* to produce and spawn mature gametes, unlike the other species of marine bivalves examined to date, may be related to the tremendous fecundity displayed by this species (Allen and Downing 1986).

Commercial Use

Even though triploid *C. virginica* are no more disease resistant than diploids (Meyers et al. 1991, this study), they may have commercial value on the east coast of the U.S. in a hatchery-based aquaculture industry, selling to a "half-shell" market. As demonstrated in this study, placing oysters into the field in late summer (August) avoided heavy *P. marinus* infection the first year. The triploids, by virtue of their greater rate of growth, reached an

average shell height equal to commercial size (63.5 mm) by July of the second year, before *P. marinus* began causing mortality and reducing growth. Diploid oysters, on the other hand, did not average commercial size until November of the second year, by which time *P. marinus* had become well established and mortalities were numerous. Almost twice as many triploids were of commercial size in July 1990 at age 15–16 months than diploids, and the meat quality of the triploids was higher than that of diploids at this time of year, owing to differences in gonadal development.

As demonstrated in this study, the timing of placement of oysters in the field is important both with respect to disease and growth. By planting late the first year (August), infection by *P. marinus* can largely be avoided and growth of triploids to commercial size can be attained by July of the second year, prior to establishment of the parasite and subsequent mortality. These relationships may be site specific, however, and further comparisons are clearly warranted.

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