2008

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EVALUATING RECRUITMENT CONTRIBUTION OF A SELECTIVELY BRED AQUACULTURE LINE OF THE OYSTER, *CRASSOSTREA VIRGINICA* USED IN RESTORATION EFFORTS

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ABSTRACT Severe over-fishing, habitat degradation, and recent disease impacts have devastated the eastern oyster (*Crassostrea virginica*) fishery in the Chesapeake Bay. Several restoration efforts are in progress, including the unconventional approach of seeding reefs with an aquaculture strain selected for disease resistance and fast growth in hopes of mitigating the negative effects of disease and low census numbers. Supplementation of four sites (The Great Wicomico, Lynnhaven, York, and Elizabeth Rivers) examined in this study totaled approximately 18,500,000 aquaculture oysters from 2002 to 2006. We collected locally recruited offspring ($n = 6517$) from 2002 to 2006 at these sites to determine if reproduction by the transplanted oysters produced detectable contributions to recruitment by examining the frequency of a composite mitochondrial haplotype that occurs at high frequencies in this aquaculture strain but is rare in wild Chesapeake Bay oysters. The estimated frequency of this haplotype in locally recruited oysters (average 1.4%, SD = 0.9) was compared with the average frequencies found in the hatchery produced (35.9%, SD = 12.8) and wild (1.2%, SD = 0.9) oysters, but we were unable to refute the null-hypothesis that population supplementation made no contribution to recruitment. We discuss five nonmutually exclusive explanations for the limited impact of supplementation, including unequal sex-ratio, predation, flushing, relative scale, and aquaculture selection. We argue that predation, relative scale, and aquaculture selection are the likely reasons for the limited contribution made by aquaculture oysters used for population supplementation.

KEY WORDS: aquaculture, *Crassostrea virginica*, eastern oyster, mtDNA, restoration, RFLP

INTRODUCTION

Many of the world's fish and shellfish stocks have been severely impacted by over-fishing and/or habitat degradation. For instance, many rivers across the northern hemisphere have been altered for hydro-electric power production, resulting in blocked upstream passage and destruction of spawning habitat for many anadromous finfish species (Cowx & Welcomme 1998). Supportive breeding programs in which local wild individuals are spawned in hatcheries and their offspring released into the wild compensate for the loss of naturally produced fish in some of these rivers (e.g., Heath et al. 2003).

The eastern oyster *Crassostrea virginica* (Gmelin, 1791) is distributed along the eastern coasts of the Americas from the Gulf of St. Lawrence in Canada, throughout the Gulf of Mexico and the Caribbean Sea, and south to Argentina (Kennedy et al. 1996). A filter feeder and reef builder, *C. virginica* is considered a keystone species in many coastal areas. Adults can reach sizes up to 20 cm (Kent 1988) and live for up to 20 y (Andrews 1979). Oysters are protandrous hermaphrodites that become female after reaching larger sizes (Thompson et al. 1996). They spawn throughout the summer, and have a feeding and actively swimming larval stage lasting for a few weeks, after which they settle on hard substrates and metamorphose into sessile adults (Andrews 1979).

The eastern oyster has suffered extensively from chronic over-fishing, habitat degradation, and mortality caused by two major parasites: *Haplosporidium nelsoni* (Haskin et al. 1966, MSX disease) and *Perkinsus marinus* (Mackin et al. 1950,

dermo disease). These impacts have severely reduced census numbers, caused the collapse of the Chesapeake Bay oyster fishery (Burreson & Andrews 1988, Rothschild et al. 1994), and motivated various restoration projects, including construction of artificial reefs using unconsolidated oyster shell and other materials. One stated restoration goal is to increase the number of oysters in the Chesapeake Bay 10-fold by 2010 over 1994 census population sizes (Chesapeake Executive Council 2002). Hatchery-propagated local oysters and dredged adult oysters have been transplanted to a number of these reefs. Early restoration efforts using wild transplants were initially considered successful but subsequent mortality, presumably caused by dermo and MSX (Southworth & Mann 1998, Southworth et al. 1999), has tempered this early optimism and prompted an unconventional approach to restoration using domesticated, genetically-improved oyster strains for seeding reconstructed reefs (Allen et al. 2003).

The use of intentionally domesticated lines subjected to artificial selection sets the oyster restoration program in Virginia considerably apart from restoration efforts in other species. Traditionally, hatchery strains used for population supplementations have been established and propagated using the offspring from a small number of initial parents, often resulting in a loss of genetic variation (Allendorf & Phelps 1980, Allendorf 1993, Launey et al. 2001, Evans et al. 2004, Taris et al. 2006) and marked divergence in allele frequencies relative to the source populations (e.g., Gross 1998) from which they are derived. To avoid intentional and unintentional hatchery selection, supportive breeding usually use rigorous hatchery

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protocols that strive to mimic natural processes and maintain a high number of breeders and thus reduce the effects of inbreeding and genetic drift (Allendorf & Phelps 1980, Frankham et al. 1986, Allendorf 1993, Ryman et al. 1995). The presumably severe effects of diseases on wild oysters have, in contrast, been used as a rationale for using intentionally domesticated lines with enhanced disease resistance for population supplementation and restoration (Allen & Hilbish 2000, Allen et al. 2003), but the advisability of this approach is the subject of considerable debate (Carlsson et al. 2006, Gaffney 2006, Hare et al. 2006).

Despite the controversy, the Army Corps of Engineers (ACOE) and the Chesapeake Bay Foundation (CBF) have adopted the use of a domesticated oyster strain (the Andrews DEBY strain, hereafter DEBY) to seed man-made reefs throughout the Virginia portion of the Chesapeake Bay. The DEBY line has been artificially selected for disease resistance and rapid growth (Ragone Calvo et al. 2003), and is one of the most popular lines for aquaculture in Virginia. Experimental seeding of reefs with DEBYs in Virginia's Great Wicomico River was initiated in 2002 to study how disease-resistant aquaculture lines would perform in restoration settings because genetic-level differences between this line and wild populations allows analyses of the reproductive success of outplanted oysters and the genetic impact of these supplementations on surrounding populations through interbreeding and introgression. As with all restoration efforts, it is crucial that prerestoration conditions are adequately documented and that postrestoration results are evaluated to allow for adaptive management. In the Great Wicomico River this is perhaps even more critical, because the use of domesticated strains represents an unusual and unorthodox approach to restoration that intentionally alters the genetic composition of wild populations whereas most supplementation efforts seek to minimize or eliminate these impacts.

Two restriction fragment-length polymorphisms (RFLP) in the COI and COIII mitochondrial genes (mtDNA) have previously shown high frequencies (composite haplotype BB) in the DEBY line relative to native Chesapeake Bay oysters (Reeb & Avise 1990, Hare et al. 2006), and these differences allow for the tracking of DEBY recruitment at restoration sites using these genetic markers. Because mtDNA is maternally inherited and not subject to crossing-over, with the exception of new mutations, it is inherited unaltered from the mother. Accordingly, mtDNA analyses can only detect DEBY contribution in spat resulting from pure DEBY mothers or DEBY female $\times$ wild male "hybrids." F_1 hybrid offspring from a DEBY female and wild male exhibit 100% DEBY mtDNA and 50% DEBY nuclear DNA (nDNA); F_2 hybrid females carrying the BB haplotype and breeding with wild males will produce offspring with 100% DEBY mtDNA but only 25% DEBY nDNA, etc. As a consequence, after several generations it becomes difficult to use the BB haplotype as evidence of recent DEBY recruitment, because 100% DEBY mtDNA might correspond to a low proportion of DEBY nDNA. Similarly, individuals not showing the BB haplotype could have a large proportion of DEBY nDNA inherited from wild male ancestors. This is a weakness and strength—mtDNA only reveals female DEBY contribution (and that is imperfect because the BB haplotype does occur in nature). On the other hand, the BB haplotype is not diluted because DEBY $\times$ wild hybrids backcross with wild oysters. Hence,

the ability to infer a historic DEBY contribution of BB haplotypes is preserved no matter when introgression took place.

The objective of the present study was to evaluate if offspring produced by DEBY oysters used to supplement four restoration sites in Virginia between 2002 and 2006 have made a detectable contribution to census numbers. We tested the null-hypothesis that there has been no detectable increase in the frequency of the BB composite mtDNA haplotype in newly recruited juveniles against the alternative hypothesis that the deployments have led to a detectable increase. Based on data from multiple sites within the Chesapeake Bay and over multiple years, this effort expands the geographic scope of genetic restoration monitoring beyond previous efforts and has the potential to find cumulative increases in DEBY contribution.

MATERIALS AND METHODS

Hatchery Samples

The DEBY strain originated from Delaware Bay wild oysters and was brought to the Virginia Institute of Marine Science in 1987, where a selective breeding program was initiated that continues to this day (Burreson 1991). The DEBY name was registered as a trademark in 2006. The main objective in establishing the strain was to create a dermo and MSX resistant stock for aquaculture (Burreson 1991, Ragone Calvo et al. 2003). DEBY oysters have a high frequency of two mtDNA haplotypes at the COI and COIII genes that are commonly found in oysters from the Gulf of Mexico but rare in Chesapeake Bay populations (Reeb & Avise 1990, Hare et al. 2006; Table 1). To estimate baseline frequencies of the BB haplotype in the hatchery-produced oysters used for population supplementation, samples of DEBYs from 7 different spawns totaling 771 individuals were collected from the Virginia Institute of Marine Sciences' hatchery between 1997 and 2005.

Restoration Sites and Sampling Methods

The Elizabeth River is a 775 km^2 estuary located in the southern Chesapeake Bay (Fig. 1). From 1998 to 2004, CBF and Oyster Reef Keepers of Virginia (ORKV) seeded several man-made reefs with approximately 460,000 oysters (average shell height >50 mm), including approximately 136,000 DEBYs. In 2004, we sampled 206 naturally-produced oysters (oysters attached to shell or other structure; shell height 7.9–106.1 mm, average = 46.3 mm, SD = 27.0) by hand or dredge from or near the seeded reefs.

The Great Wicomico River is a small, 337 km^2 trap-like estuary (Andrews 1979, United States Army Corps of Engineers 2003) in the southwestern Chesapeake Bay. Between 1996 and 2006 some 17,300,000 oysters were either translocated to these reefs from other wild populations or seeded using aquaculture strains, including approximately 15,500,000 DEBYs (Table 1), most of which (>90%) were planted on a single reef. We collected a sample of 128 wild adult oysters (Table 1) prior to the first DEBY deployment in 2002. Since then, we have sampled naturally produced spat annually at six to seven sites in the river surrounding the reef using wire mesh bags filled with oyster shell (0.02 m^3) (Hare et al. 2006). The shell bags were collected and replaced bimonthly from June to October between 2002 and 2006. Shells were screened for spat by eye and individual spat (<20 mm) were collected and preserved in

TABLE 1.

The number of deployed domesticated oysters and the number and proportion of haplotypes found in spat, wild, and domesticated oysters from four Virginia river systems.

Location	Deployment Dates	#DEBY Deployed	Collection Dates	n	Haplotypes				Fisher's Exact Test (two tailed P)		
					AA	BB	Rare	%BB	R125/3*	R1416/16*	R1541/19*
Elizabeth R.	2002–04	136,000	2005	204	199	4	1	1.96	1.000	0.299	0.328
Lynnhaven R.	2002–04	755,800	2005	932	920	4	8	0.43	0.042	0.106	0.052
York R.	2003	98,000	2005	92	91	0	1	0.00	0.268	0.619	0.620
Great Wicomico R. wild		0	2002	128	125	3	0	2.34	n/a	n/a	n/a
Great Wicomico R.	2002	795,700	2002	1,281	1,259	9	13	0.70	0.089	0.317	0.187
Great Wicomico R.	2003	292,060	2003	286	282	1	3	0.35	0.092	0.336	0.345
Great Wicomico R.	2004	1,410,000	2004	109	109	0	0	0.00	0.252	0.622	0.630
Great Wicomico R.	2005	6,071,648	2005	889	857	19	13	2.14	0.753	0.053	0.088
Great Wicomico R.	2006	6,928,352	2006	2,721	2,648	52	21	1.91	0.737	0.054	0.084
Great Wicomico R. combined	2002–06	15,497,760	2002–06	5,286	5,155	81	50	1.53	0.454	0.263	0.402
All rivers	2002–06	18,487,560	2002–06	6,517	6,368	89	60	1.37			
Hatchery			1999	60	29	31	0	51.67			
Hatchery			2002	83	45	36	2	43.37			
Hatchery			2003	139	95	41	3	29.50			
Hatchery			2004	244	125	112	7	45.90			
Hatchery			2005	171	98	69	4	40.35			
Hatchery			2005	24	20	4	0	16.67			
Hatchery			2005	50	38	12	0	24.00			
Hatchery combined			1999–05	771	450	305	16	35.92			

* The ratio of AA and BB haplotypes that the observed ratio was compared with Fisher's Exact Test.

ethanol for subsequent genetic analysis. Between 2002 and 2006, 5,286 spat were collected and analyzed.

The Lynnhaven River is a small estuary of 166 km^2 consisting of three branches that join at the Lynnhaven inlet. Between 1998 and 2004, approximately 1,560,000 oysters were deployed by CBF and ORKV, including some 756,000 DEBY oysters between 2002 and 2004 (average shell height >50 mm). A total of 932 oysters (shell height range = 5.1–151.4 mm, average = 59.9 mm, SD = 30.6, n = 799) were collected by hand in 2004 from several man-made reefs that had received DEBYs, and from a number of unseeded locations (Table 1).

The York River is a major tributary (watershed size 4,350 km^2) of the Chesapeake Bay. In 1998 the Virginia Marine Resource Commission (VMRC) constructed an artificial reef near Felgates Creek, which was seeded between 2000 and 2004 by CBF and ORKV with 352,000 oysters from several strains, including 98,000 DEBYs (average shell height >50 mm). Ninety-two naturally produced oysters (shell height range = 54.6–120.1 mm. average = 88.1 mm, SD = 15.4, n = 60) were sampled by hand from the reef in 2004 (Table 1).

In addition to the restoration efforts mentioned earlier, some DEBY deployments occurred from late 2001 through 2004 in other Chesapeake Bay tributaries. These were all smaller deployments (excluding one deployment of 385,000 oysters in the Piankatank River, VA) and totaled approximately 230,000 oysters spread over 6 rivers. No samples were taken from these deployments.

Genetic Analyses

Whole genomic DNA was extracted from individual oysters using the DNeasy Tissue Kit (Qiagen Inc., Santa Clara, CA,

USA) according to the manufacturers' instructions and stored at –20°C. Polymerase Chain Reactions (PCR) amplifications of the COI gene region were carried out using primers (forward: 5′-GCT GTT ATG TCC ACT AAT CAT C TTG- 3′, reverse: 5′ ACT GGG TCA CCA CCA CCT AC 3′) modified from Folmer et al. (1994). Amplifications consisted of 15 µL reactions containing 11.46 µL sterile dH$_2$O, 1.5 µL 10 × PCR buffer, 0.60 µL 50 mM MgCl$_2$, 0.30 µL 10 mM each dNTPs, 0.01 µL 100 µM forward and reverse primers, 0.12 µL *Taq* I polymerase (0.60 U total), and 1 µL DNA template (approximately 25 ng DNA total). PCR amplifications of the COIII gene region used the primers (forward: 5′- ATT TAG TTG ATC CTA GGC CTT G-3′; reverse: 5′-ACT CAA ACC ACA TCT ACA AAA T-3′) of Milbury (2003). Amplifications consisted of 15 µL reactions containing 11.51 µL sterile dH$_2$O, 1.5 µL 10 × PCR buffer, 0.60 µL 50 mM MgCl$_2$, 0.30 µL 10 mM each dNTPs, 0.0075 µL 100 µM forward and reverse primers, 0.075 µL *Taq* I polymerase (0.375 U total), and 1 µL DNA template (approximately 25 ng DNA). The COI and COIII PCR amplifications were performed with initial denaturation for 7 min at 95°C, followed by 45 cycles of 1 min at 95°C, 1 min at 45°C, 2 min 30 sec at 72°C, and 7 min final extension at 72°C.

Amplified products were digested with restriction enzymes *Hae*III (COI) and *Hinf* I (COIII) following manufacturer protocols (New England Biolabs, Inc., Beverly, MA, USA), The digested products were separated by electrophoresis on 1.5% (2:1 agarose: low melt agarose; Fisher Scientific) gels in × 1 TBE buffer. Gels were stained in 0.5 µg/mL EtBr baths, visualized using a UV transilluminator, and recorded with AlphaImager 5.5 imaging software (Alpha Innotech Co., San Leandro, CA, USA).
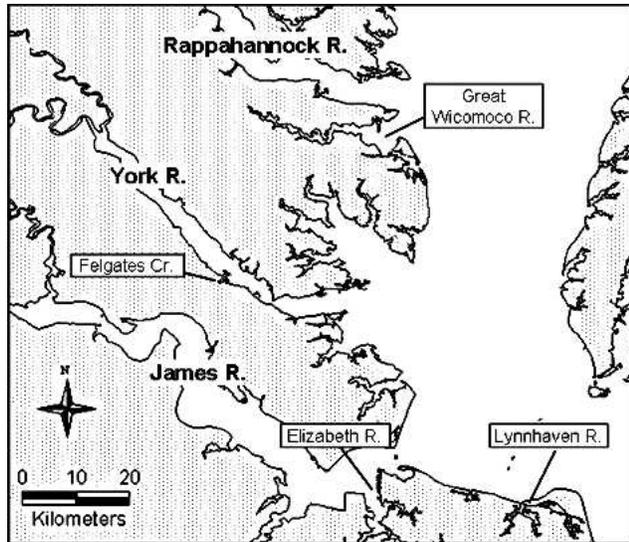
Figure 1. Location of four restoration sites that have received DEBY transplants in the Virginia portion of the Chesapeake Bay.

Tests for the Influence of Deployed Hatchery Oysters

To test the null-hypothesis that there has been no detectable increase in the BB composite mtDNA haplotype in Great Wicomico River annual spatfalls, we compared postdeployment composite haplotype frequencies to frequencies derived from various predeployment (wild) samples using Fisher Exact Test. Predeployment frequencies were calculated based on (1) our 2002 Great Wicomico River sample ($n = 128$) taken prior to DEBY deployments, (2) Great Wicomico River samples ($n = 1558$) collected in 2002 by Hare et al. (2006) and determined to be of wild parentage based on mtDNA and microsatellite analyses, and (3) the pooled samples from these two data sets. Other work in this laboratory (KSR, unpublished data), has shown similar BB haplotype frequencies in other wild *C. virginica* populations. Because predeployment samples were not available for the remaining restoration sites (Elizabeth, Lynnhaven and York Rivers), we used the three estimates of predeployment BB haplotype frequencies mentioned earlier for comparison with postdeployment frequencies at the remaining sites.

Oyster Sex Ratios

Because mtDNA markers trace only the maternal contribution to spawning, a sex ratio skewed towards males in the deployed oysters could limit the usefulness of this class of markers. Oyster sex ratios were obtained during routine histopathological evaluation of hatchery-produced DEBYs from groups deployed to the GWR restoration sites. Eight samples ($n = 25–57$) of oysters destined for deployment it the Great Wicomico River were obtained from oyster farms on the York River between October 2003 and May 2006 (Table 2). Oysters were measured (shell height, ±0.1 mm), shucked, and soft tissues were fixed as a group in Davidson's Fixative (Shaw & Battle 1957). Tissues were processed histologically using standard methods (e.g., Burreson et al. 1988). Oyster sex was noted during microscopic parasitological analysis of hematoxylin and eosin-stained slides. Divergence from expected 1:1 sex ratios were evaluated using Fisher Exact Tests.

RESULTS

Prior to any DEBY deployment, a sample of 128 oysters collected in 2002 from the Great Wicomico River contained 3 BB haplotypes, equivalent to a frequency of 2.3% (Table 1). The spat sample from 2002 analyzed by Hare et al. (2006) using combined microsatellite and mtDNA data was larger (1,558 individuals). In their analysis they identified 142 DEBY × wild F_1 hybrids. Twenty-one individuals had the BB haplotypes, and after removal of F_1 hybrids from the dataset there were 16 BB haplotypes (1.1%) in 1,416 spat of presumed wild parentage. Pooling these two "wild" samples, we estimated the BB frequency in spat and adults from the GWR in 2002 as (16 + 3)/(1,416 + 128) = 1.2%. The BB haplotype frequency in the hatchery spawned DEBYs was considerably higher, averaging 35.9% (SD = 12.8, Table 1).

Approximately 18,500,000 DEBYs were deployed in the four river systems between 2002 and 2006 (Table 1). We genotyped 6,517 spat during this time period at the COI and COIII genes and found 89 BB haplotypes (1.4%). Of these, 81 (1.5%) were found in the combined samples of 5,286 spat from the Great Wicomico River between 2002 and 2006, 4 of 207 (2.0%) were found among the oysters collected from the Elizabeth River, 4 of 932 (0.4%) were found in the Lynnhaven River, and none were found among the 92 oysters from the York River. BB haplotype frequencies did not differ from the expected ratios in any river (Fisher's Exact Test $P > 0.05$ for all tests, except the Lynnhaven River that showed a reduced BB ratio after DEBY deployments, Table 1). Note that the Great Wicomico River data from 2002 through 2006 are not independent, as the observed ratio of haplotypes in any year is affected by spat contributions from previous deployments.

If the deployed DEBYs had contributed to the annual spatfall in the Great Wicomico River, it should have led to a cumulative increase in the BB haplotype frequency over time, as this river has received annual supplementations of DEBYs since 2002 (Table 1), with a cumulative deployment of more than 15,000,000 oysters (approximately 84% of the total deployment) between 2005 and 2006. To date, however, no significant increase of BB frequency could be found when correlating time in years with cumulative BB frequency in the Great Wicomico River ($n = 6$, Spearman Rank Order Correlation, corrected correlation = –0.143, $P = 0.749$).

Gender could be determined for 179 of 242 DEBY oysters destined for the Great Wicomico River and evaluated histologically (average = 22.4 oysters/sample). The proportion of females ranged from 18.2% to 90.0% among samples (mean = 55.1%, Table 2) and three oysters were hermaphrodites. The sex ratio was significantly skewed in favor of females in two samples (May 12, 2005; and Jul. 6, 2005); and in favor of females in three (May 25, 2004; May 12, 2005; and Jul. 6, 2005). Sex ratios in the remaining samples were not significantly divergent from 1:1. Samples skewed toward females tended to contain larger individuals, though the sample with the second largest average size (Oct. 10, 2003; 75.5 mm, SD = 8.4 mm) contained more males (although not significantly). Among samples containing the smallest average sizes

TABLE 2.

Oyster sex ratio data from samples of DEBY oysters bound for restoration sites in Great Wicomico River.

Date	n	Size (range, mm)	Size (mean ± SD, mm)	No. Sexed #	Female #	Female %	Male #	Male %	Herm. #	Herm. %	Fisher's Exact Test Two-tailed P
Oct. 10, 2003	30	63.8–96.0	75.5 ± 8.4	11	2	18.2	9	81.8	0	0.0	0.183
May 25, 2004	57	37.5–97.5	59.1 ± 16.7	52	34	65.4	18	34.6	0	0.0	0.164
Apr. 25, 2005	30	36.7–66.1	48.9 ± 8.9	14	6	42.9	7	50.0	1	7.1	1.000
May 12, 2005	25	60.4–83.9	71.0 ± 6.5	20	18	90.0	1	5.0	1	5.0	0.003
Jul. 6, 2005	25	62.3–92.2	75.3 ± 9.3	25	20	80.0	5	20.0	0	0.0	0.038
Jul. 11, 2005	25	43.4–74.8	56.2 ± 7.7	24	13	54.2	11	45.8	0	0.0	1.000
Sep. 19, 2005	25	75.7–96.5	87.0 ± 6.3	10	6	60.0	4	40.0	0	0.0	0.999
Nov. 5, 2006	25	36.9–62.0	47.7 ± 5.3	23	7	30.4	15	65.2	1	4.3	0.358

(47.7–59.1 mm) the average sex ratio was not significantly divergent from 1:1.

DISCUSSION

The stated goal for oyster restoration in the Chesapeake Bay is a 10-fold increase in the number of native oysters by 2010 compared with the 1994 census size (Chesapeake Executive Council 2002). We focused on the question of whether the DEBY deployments have made a detectable contribution to spat recruitment to date in four Chesapeake Bay river systems. In this context, the lack of significant changes in BB frequency attributable to spat produced by the outplanted DEBY oysters would be considered inadequate to meet the stated goal. Given the high frequency of the BB haplotype in DEBY spawns (average 35.9%) and the low frequency in wild oysters (1.2% based on pooled data), we would expect an increase in the BB frequency in wild-produced spat if the DEBY strain was a major contributor to the breeding population. Only three samples have BB frequencies higher than 1.2%: the 2005 (2.1%) and 2006 (1.9%) Great Wicomico River samples, and the Elizabeth River sample (2.0%). These small changes in frequencies (<1.0% increase) could be well within expectations from natural fluctuations of alleles caused by genetic drift and/or sampling errors. Unfortunately, we cannot rigorously test if the changes in BB haplotype frequency are significantly different from zero, because we have no information about the frequency of BB haplotypes prior to DEBY deployments in any river except for the Great Wicomico River. However, by assuming similar AA and BB haplotype background ratios in the remaining study sites, the ratio of AA and BB haplotypes were not significantly different from prior to DEBY deployments (see Fisher's Exact tests in Table 1).

Assuming that the DEBY oysters made some small (albeit statistically nonsignificant) contribution to recruitment, it is of some interest to estimate the potential proportion of DEBY spat resulting from the deployments. To estimate the frequency of BB haplotypes that could be caused by DEBY reproduction (offspring from pure DEBY × DEBY and DEBY females × wild males crosses) we subtracted the natural background BB haplotype frequency from the observed frequency of BB haplotypes to obtain an estimate of the increase in the BB haplotypes that is potentially attributable to DEBY reproduction. However, because only 35.9% (on average) of the DEBY oysters carried the BB haplotype, this estimate represents only spat from DEBY individuals carrying the BB haplotype. Hence, we divided this frequency by the average frequency of BB haplotypes observed in the DEBY oysters deployed to estimate the total proportion of spat that might have originated from DEBY reproduction. Note that the tests and estimates assume that the frequencies of BB haplotypes in all sampled locations are very similar to the observed values from the Great Wicomico River. Assuming an average BB haplotype frequency of 1.2% in Chesapeake Bay wild populations and 35.9% in the DEBY spawns, we estimate the proportion of pure bred and maternal hybrid DEBY spat produced in the Great Wicomico River, pooled over years as 0.8%, with annual estimates varying from 0.0% to 2.5%. Similar estimates for the York River and Lynnhaven River samples were 0.0% and 2.2% in the Elizabeth River. The average proportion of DEBY derived spat across all rivers was estimated at 0.6%.

Hare et al. (2006) estimated that approximately 10% of the 2002 spat fall in the Great Wicomico River consisted of DEBY hybrids based on nuclear microsatellite and mtDNA data. The contribution is closer to 5% after accounting for type I errors (M.P. Hare unpublished data). Though comparison of results derived from nuclear *versus* mitochondrial DNA is not straightforward, Hare's microsatellite-based estimate is similar to ours based on mtDNA markers. For instance, the highest proportion of BB haplotypes found in the present study was in the 2005 Great Wicomico River sample (2.1%; assuming a wild background BB frequency of 1.2% and an average frequency in deployed DEBYs of 35.9%, this is equivalent to 2.5% DEBY pure bred and maternal hybrids). Assuming that the sex ratio was 1:1 and that no pure-bred DEBYs were present among our spat samples (Hare et al. 2006 observed mostly F_1 DEBY × wild hybrids and only one pure-bred DEBY spat), our mtDNA observations imply an overall contribution of 5% (2.5% maternal and 2.5% paternal hybrids). Consequently, oysters other than the DEBY strain must be responsible for the most production in these four river systems. We are not aware of any quantitative goals for the proportional contribution desired from supportive breeding with DEBY oysters. Our estimate of 5% suggests, on the one hand, a level of recruitment enhancement that could have a large impact on populations over the long term. However, we did not observe any cumulative increase in the frequency of BB haplotypes so it is possible that survival to reproduction was low and our measures based on spat are inflated relative to proportional reproductive

contributions. We discuss five factors, not mutually exclusive, that might have limited the contribution of the deployed DEBY strain to date.

Unequal Sex Ratio

Oysters are protandrous hermaphrodites, becoming female after reaching larger sizes (Thompson et al. 1996). Our mtDNA data could not trace male reproduction, so if the deployed oysters were mainly males, we would have underestimated their spawning contribution. The only data bearing on this possibility come from Hare et al. (2006) wherein mtDNA and eight microsatellite loci were used to assign "wild" spat collected in the Great Wicomico River in 2002 to either wild oysters or DEBY brood stock planted June to July of the same year. DEBY oysters planted in 2002 had average shell length of 64.1 mm (range 47.7–87.2 mm), large enough to be reproductive but with an unknown sex ratio. Indeed, 153 of the 1579 analyzed spat had multilocus genotypes identifiable as F_1 progeny of DEBY × wild oyster crosses (Hare et al. 2006). The production of F_1 hybrids implies that DEBY oysters had a skewed sex ratio or low fecundity so that coexisting wild oysters achieved most fertilizations. Given that the BB haplotype frequency was 45.0% among 2002 DEBY brood stock and 0.5% in wild Chesapeake oysters, F_1 spat produced by equal numbers of male and female DEBYs had an expected BB frequency of ~23.0%. Instead, among the 142 Great Wicomico River recruits determined to be F_1 for which mtDNA data existed, the frequency of BB haplotypes was 3.5% (M.P. Hare unpublished data), confirming that most DEBYs bred as males to produce the F_1 spat.

Burkenroad (1931) reported that sex reversal usually takes place at a total length of 40 mm, whereas Coe (1932) reported that sex ratios close to 1:1 were reached at sizes of >50 mm. Numerous studies have indicated that sex ratios can change rapidly (Thompson et al. 1996), and Kennedy (1983) showed experimentally that individuals in unisexual groups of oysters would change sex over time such that 1:1 ratios would tend to be restored. The average size of the oysters transplanted to the GWR ranged from 47.7–87.2 mm (c.f., Table 2). The lowest female to male ratio observed in oysters en route to the Great Wicomico River over the last few years was ~2:9, in a sample from 2003. The average has been ~1:1 but there is a large amount of variation. The lack of a consistent male bias and the propensity of oysters to change sex make it unlikely that an unequal sex ratio (i.e., a preponderance of males) has been a major cause of the apparently limited DEBY contribution.

Predation

To date, all DEBY deployments in the four river systems studied have used cultch-less oysters because they are simple to manage in aquaculture situations. Unfortunately, cultch-less spat are highly susceptible to predation, and significant predation by cow-nosed rays (Rhinoptera bonasus, Mitchill, 1815) and blue crabs (Callinectes sapidus, Rathbun, 1896) at a number of deployment sites including the Great Wicomico River has been reported anecdotally in the popular press. It is possible that predation has been so severe that few deployed oysters survived to reproductive age. In contrast to cultchless oysters, naturally produced spat attached to hard substrate are, to some extent, protected from predators (Allen et al. 2003). In an attempt to mimic natural spat production, a new deployment strategy was implemented in Virginia in 2005–2006. Currently, efforts are being made to set domesticated spat on shell in the hatchery and then transplant them to deployment sites (Allen et al. 2003), but the success of this strategy remains to be evaluated. Because of temporal and spatial variability in predation intensity, it is difficult to determine whether predation is limiting the reproductive contribution of deployed oysters equally across restoration sites. Nevertheless, it is conceivable that predation could be a major factor limiting the contribution of deployed oysters.

Flushing

Oyster offspring predominantly recruit locally to reefs or river systems (Rose et al. 2006, North et al. 2008). Deviations from this assumption could be caused by tidal regimes or climatic events such as heavy rains and wind-driven storms that occur during the planktonic phase, resulting in larvae being flushed from the system (c.f., Hare et al. 2006). Because sampling in the Elizabeth, Great Wicomico, and York Rivers were conducted on or near the deployment sites, increased flows might have flushed locally produced larvae away from our sampling areas or even out of the systems preventing us from detecting a genetic signal from deployed DEBYs. Whereas the argument is plausible for the York River (because it is not a trap estuary), it is less likely for the more widely sampled Lynnhaven River system, because several locations outside the deployment areas were sampled.

Oyster larvae have a relatively short pelagic phase that lasts up to two weeks, during which they passively or actively swim towards the bottom in connection with tidal flows (Turner et al. 1994). It has been suggested that this behavior could be an adaptation for retention (Finelli & Wethey 2003). If heavy rains cause floods that flush oyster larvae out of these river systems, they must be strong enough to affect larvae low in the water column. To explain our results, these floods would have had to occur during the larvae phase each year the DEBYs reproduced in all sampled river systems. Note that one main reason that the Great Wicomico River was chosen for experimental restoration was that it shows strong retention that should enhance larval retention (Southworth & Mann 1998). Given the likelihood that strong floods coinciding with peak larvae pulses occurred each year at every deployment site sampled, we argue that flushing is not a consistent explanation for the limited DEBY spat contribution observed.

Relative Scale

The reproductive contribution of the deployed DEBYs might be swamped by the reproduction of more numerous local oysters. There are no reliable estimates of local census sizes at these restoration sites prior to deployment except for the Great Wicomico River, which was estimated to support 10,000,000–15,000,000 adult oysters in recent years (R. Mann, VA Institute of Marine Science, Personal communications). The Great Wicomico River also received the largest number of DEBYs (15,500,000 cumulatively, Table 1). All four systems were selected for deployment, because they were believed to harbor few remaining natural oysters. Note that the two largest annual deployments of DEBYs in the Great Wicomico River occurred in late 2005 and through the spring, summer, and fall of 2006,

and future monitoring may detect larger DEBY reproductive contributions. In addition, all four sites have received deployments of domesticated oysters of other strains than DEBY prior to the DEBY deployments. We cannot track the reproductive contribution of oysters other than DEBYs with the genetic methods used in this study. Future studies using other genetic markers (e.g., microsatellites) might have the resolution to resolve the reproductive contribution of these deployments. We suggest the possibility that local, predeployment census sizes were under-estimated and/or that the numbers of reproductively active deployed DEBY oysters were inadequate to detect DEBY recruitment.

Aquaculture Selection

The DEBY strain has been selected for rapid growth and disease resistance under aquaculture conditions (Burreson 1991, Ragone Calvo et al. 2003), and its performance under natural conditions is not well documented. Likewise, studies focusing on the performance of other domesticated molluscan strains under natural conditions are generally lacking. A considerable body of data is, however, available from studies of salmonids. Biologists have argued that artificial selection for survival and growth in artificial environments such as hatcheries and net-pens is different from natural selection under conditions encountered in the wild (Gross 1998). As early as the 1860s Darwin (1868) pointed out that unintended selection for survival and reproduction traits in captivity is inevitable. In fact, many biologists consider domesticated conspecifics as a major threat to wild populations (e.g., Heggberget et al. 1993). Even supportive breeding efforts to supplement wild populations have been criticized because unintentional selection can lead to rapid, negative changes in essential life-history traits (Gross 1998, Heath et al. 2003, Araki et al. 2007). Hybrids between domesticated and wild salmon express lower fitness than their wild counterparts (e.g., McGinnity et al. 2003). The loss of locally adapted genes and gene complexes in wild populations through genetic pollution from domesticated stocks is considered to be a major threat to wild salmon (Gross 1998, Myers et al. 2004). Unfortunately, there are no studies of fitness differences between wild and domesticated oysters or their hybrids under natural conditions. It is likely, however, that the artificial selection observed in farmed salmonids is also operating in hatchery propagation of oysters. Artificial selection for disease resistance and growth is likely to be accompanied by unintentional selection for nontargeted traits, and may reduce fitness in unmanaged environments. Consequently, we argue that selective breeding and the effects of unintentional selection associated with hatchery propagation of the DEBY strain could have lead to reduced fitness in the wild, and might be a major cause for the lack of substantial reproductive contribution of deployed DEBY oysters.

CONCLUSION

There have been significant deployments of hatchery-propagated DEBY oysters in several Virginia estuaries adjacent to the Chesapeake Bay, yet to date we have been unable to detect a significant DEBY contribution to wild-produced spat. We hypothesize that contributions to recruitment by DEBY oysters have been low for three primary reasons: (1) predation could have decimated the deployed oysters before they could reproduce, (2) census numbers of wild oysters may have been underestimated and too few DEBYs were deployed to expect an observable contribution, and (3) DEBYs have low fitness under natural conditions caused by aquaculture selection. The recently proposed approach of deploying spat on shell instead of cultchless oysters could mitigate predation to some extent and may allow more deployed oysters to breed. This approach, however, remains to be evaluated under experimental conditions. On the other hand, spat on shell will not alleviate the problems associated with hatchery adaptation or improve the fitness of DEBYs under wild conditions. We urge that potential fitness differences between aquaculture lines and local wild oysters be evaluated in common garden settings or similar experiments. Such information is essential for developing optimal restoration strategies and would improve adaptive management.

ACKNOWLEDGMENTS

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