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ARTICLE

Genetic Evaluation of Supplementation-Assisted American Shad Restoration in the James River, Virginia

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Abstract

Hatchery supplementation programs have been implemented for several populations of American Shad Alosa sapidissima, which are declining across the species’ native range due to disrupted access to spawning grounds, habitat degradation, and overfishing. The genetic impacts of stocking Pamunkey River-origin larvae into the James River American Shad population since 1994 were investigated, and the effects were considered within a regional context by including American Shad populations from other Chesapeake Bay tributaries that also received interbasin stockings from various rivers over the same period. Levels of genetic diversity for microsatellite markers were high in all populations except the Susquehanna River population, which showed a significant decline in diversity between the 1990s and 2007. Before supplementation of James River American Shad, the James and Pamunkey River populations exhibited subtle standardized differentiation among groups ($F_{CT} = 0.012$), whereas differentiation was reduced after supplementation ($F_{CT} = 0.007$), indicating that supplementation contributed to homogenization of population structure within the two rivers. Chesapeake Bay tributaries also displayed higher levels of differentiation in the 1990s ($F_{CT} = 0.063$) than in contemporary, supplemented samples ($F_{CT} = 0.004$). Bayesian analyses of population structure among 1990s Chesapeake Bay samples only identified the Susquehanna River as having a distinguishable population, and no population structure was detected among samples collected in the late 2000s. In light of the fact that Chesapeake Bay American Shad populations are not rebounding in response to supplementation, our observation of reduced genetic differentiation among populations is a likely signal of substitution by hatchery-origin fish rather than increasing natural recruitment. As such, spawning habitat improvement in conjunction with continued baywide fishing regulation may be a more beneficial strategy for restoring viable American Shad populations than continued reliance on supplementation.

The American Shad Alosa sapidissima is an anadromous alosine clupeid with a North American native range extending from the Saint Johns River, Florida, to the Saint Lawrence River, Quebec (Leim 1924). American Shad populations within the species’ native range are collectively at their lowest levels in recorded history due to the combined effects of overfishing, pollution, and a lack of access to spawning habitat from dam construction (ASMFC 2007; Limburg and Waldman 2009). As

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a result, numerous restoration programs have been initiated in multiple states, with the common goal of creating self-sustaining populations through harvest regulation, hatchery supplementation, and re-establishment of access to historical spawning grounds via dam removal or the construction of fish passage facilities (ASMFC 2007). Unfortunately, despite these efforts, few populations have shown persistent improvement, thus calling into question the effectiveness of practices such as hatchery-based supplementation for restoration initiatives (Hasselman and Limburg 2012).

Hatchery-based supplementation is the primary emphasis of most contemporary American Shad restoration programs and is intended to re-establish extirpated runs and supplement natural reproduction in depressed populations (Hendricks 2003; Hasselman and Limburg 2012; Moyer and Williams 2012; Bailey and Zydlewski 2013). The goal of American Shad supplementation is augmentation of wild spawning populations, which will then provide greater harvest opportunity. Based on the general consensus that homing fidelity in American Shad is on the order of 90% (Melvin et al. 1986; Dadswell et al. 1987; Walther et al. 2008), supplementation initiatives are river specific, with the expectation that stocked larvae will return to the same river as adults. The success of American Shad supplementation is traditionally gauged by tracking the proportion of hatchery versus wild individuals over time; this is accomplished by screening the returning recruits for otolith oxytetracycline (OTC) marks created in the hatchery. However, OTC tags provide limited power to analyze other population characteristics that may be impacted by supplementation, such as genetic diversity, population structure, and effective population size (Utter 1998; Fraser 2008; Moyer and Williams 2012; but see Brown et al. 1997 for an example of the concurrence between physical and genetic tags in estimating the proportion of stocks contributing to mixed-stock fisheries). In contrast to physical tags, molecular genetic markers are useful tools for investigating population genetic processes, and they provide results for tagged and untagged specimens (Brown et al. 1999; Schwartz et al. 2007). Although an extensive body of literature documents the genetic impacts of supplementation on Pacific salmonids (see Fraser 2008 for a review), robust evaluations for other supplemented species, including American Shad, are limited. Ultimately, there is scant evidence to suggest that American Shad populations are immune to the same potential consequences of supplementation (Hasselman and Limburg 2012). Therefore, an investigation of the effects and effectiveness of supplementation on American Shad populations from a genetic perspective is timely and warranted given the continued and increasing use of supplementation as a restoration tool.

American Shad supplementation along the Atlantic coast has a history dating back as far as the 1860s, and the proliferation of these hatcheries from the 1870s to the 1900s was due to the prevailing view of the period: that extensive supplementation of American Shad larvae could offset declining catches (Mansueti and Kolb 1953). Some of the largest hatcheries were located in tributaries of Chesapeake Bay, including the Susquehanna and Potomac rivers, where the number of eggs collected and number of fry stocked were staggering in comparison with contemporary hatchery outputs. From 1872 to 1949, the federal government stocked more than $4 \times 10^9$ American Shad fry into rivers along the U.S. Atlantic coast (Hendricks 2003); this number does not include fry stocked by tribal and state governments. Some of the most intensive supplementation of American Shad populations in the species’ native range has been within Chesapeake Bay tributaries (Mansueti and Kolb 1953; ASMFC 2007), especially the Susquehanna River, in which supplementation was resumed in the 1970s and has included larvae from broodstock collected in the Columbia River, Chesapeake Bay rivers, and the Delaware, Hudson, and Connecticut rivers (St. Pierre 2003). Despite extensive supplementation, precipitous declines in relative abundance from the 1950s through the 1980s prompted the 1994 imposition of a fishing moratorium throughout Chesapeake Bay and its tributaries (ASMFC 1999); however, it is worth noting that obvious declines in the Chesapeake Bay fishery began in the late 1800s (Limburg and Waldman 2009). Since the early 1990s, Virginia has initiated large-scale American Shad restoration efforts, and Maryland has expanded American Shad restoration and supplementation beyond the Susquehanna River and its tributaries (Hendricks 2003; Olney et al. 2003). These restoration efforts include hatchery components (Supplementary Table S.1) in addition to habitat improvements and fishing regulation. For example, the Potomac River has been stocked with Potomac River-origin larvae since 1995. The Nanticoke River was stocked initially with Nanticoke River-origin larvae in 1995 but later received stockings from the Potomac and Susquehanna rivers. The Patuxent River was originally stocked with Connecticut River larvae in 1993 but later received primarily Susquehanna River-origin stockings. The Rappahannock River has been stocked with Potomac River-origin larvae since 2003. Although some of these restoration programs considered genetic relationships among river populations in their design and implementation (e.g., stocking of the James River with Pamunkey River-origin fry; Brown et al. 2000), most have ignored the genetic relationships of source and recipient populations, despite the knowledge that if source and recipient populations show appreciable genetic differentiation, artificial mixing of the divergent stocks may result in outbreeding depression or the loss of unique adaptive variability (Utter and Epifanio 2002; Fraser 2008; Hasselman and Limburg 2012). In addition, stock transfers have the potential to homogenize population structure that once was detectable among some Chesapeake Bay populations (Epifanio et al. 1995; Waters et al. 2000).

Aside from the Susquehanna River, the most intensively supplemented Chesapeake Bay population of American Shad since the 1990s is the James River population (Supplementary Table S.1). Since 1994, millions of hatchery-reared larvae obtained from the Pamunkey River (a tributary of the York River; Figure 1) have been stocked annually into the James River above Bosher’s Dam (Supplementary Table S.1; VDGIF 2009). All larvae stocked in the James River since 1994 have been marked
with an OTC otolith tag at the hatchery, allowing identification of adult fish as being of hatchery origin (with OTC tag) or natural spawning origin (without OTC tag). Some of the Pamunkey River-origin larvae are concurrently stocked back into the Pamunkey River. In addition, the Pamunkey Tribal Government has operated an American Shad hatchery since 1918 on the Pamunkey River using only Pamunkey River broodstock, with all larvae being stocked back into the Pamunkey River. To monitor recruitment of hatchery fish in the James River, the Virginia Department of Game and Inland Fisheries (VDGIF) collected yearly samples of American Shad from the James River spawning grounds for analysis of OTC percentages from 1994 to 2009. The VDGIF monitoring data showed an overall high proportion of hatchery-origin recruits from 1998 to 2002, after which the number of fish with OTC marks declined (Figure 2). A secondary goal was to compare any observable shifts in genetic diversity resulting from supplementation of the James River American Shad population with possible population structure changes in other major Chesapeake Bay river populations, some of which also have been heavily supplemented (Supplementary Table S.1) and most of which also have experienced precipitous declines.

Samples collected during the 1990s and contemporary samples collected in 2006–2009 were characterized to provide insight into whether extensive supplementation since the 1990s has changed American Shad population structure among Chesapeake Bay tributaries.

**METHODS**

**Sample collection.**—Samples were assigned a priori to populations by capture location (Table 1; Figure 1); year-specific collections are designated herein by the first three letters of the river name and two digits corresponding to the year of collection (e.g., “Jam93” for the James River in 1993, “Pot00” for the Potomac River in 2000, “Rap08” for the Rappahannock River in 2008, etc.). Samples collected from the Pamunkey and James rivers during 1992–1996 were considered “pre-supplementation” samples, defined as those collected prior to the first detection of Pamunkey River-origin adults from supplementation in the James River (i.e., in 1997). Other Chesapeake Bay tributary samples collected during 1992–1993 were also considered pre-supplementation, with the exception of Susquehanna River samples. A pre-supplementation classification for
the Susquehanna River population from the 1990s is not possible because this population has an extensive contemporary supplementation history (i.e., not considering the 1800s and early 1900s supplementation efforts) dating to the 1970s (St. Pierre 2003). Therefore, we refer to 1992–1993 samples from the Susquehanna River as “early Susquehanna River.” Other samples referred to as “post-supplementation” were those collected in any Chesapeake Bay tributary in the year 2006 and later. It is important to acknowledge the caveat that even our earliest collections are truly “post-supplementation” due to the intensive stocking initiated in the late 1800s throughout Chesapeake Bay. Therefore, past supplementation may already have influenced population structure among our earliest samples; nevertheless, our focus on samples collected from tributaries over the period 1992–2009 yields valuable information about the impacts of supplementation on the contemporary population structure of Chesapeake Bay American Shad. In addition, population-relevant tissue samples taken from American Shad prior to the 1990s are (to our knowledge) not available. Therefore, our 1990s samples are the best available for discerning impacts of recent supplementation. With one exception (described below), all samples were collected from adult American Shad that were captured on the spawning grounds within each river to maximize the chance of sampling fish originating from that natural river (Epifanio et al. 1995). Because no pre-supplementation adult American Shad were available from the Potomac River, 19 juveniles collected by VDGIF in 1993 were analyzed.

Extraction of DNA.—Tissues used for DNA extraction were muscle or fin clips preserved in an 80% solution of ethanol or isopropanol; dried scales from scale envelopes that were left to dry at room temperature; and DNA that was isolated previously by Epifanio et al. (1995) and Brown et al. (1996, 2000). Portions of DNA extracts were diluted 1:10 for subsequent PCR. The PCR amplicons were fluorescently labeled with FAM, HEX, or TET reporter dyes either through direct labeling of the shorter member of each primer pair with a 5′-end fluorescent tag or by modifying the 5′ end of the shorter primer to include a universal tail (5′-CAGTCGGCGTACATCA-3′), as described by Boutin-Ganache et al. (2001), to incorporate the chosen reporter dye during PCR.

Microsatellite loci and genotyping.—All American Shad samples were genotyped at nine microsatellite loci: Asa-4, Asa-6, Asa-8, Asa-9 (Waters et al. 2000); AsaB020, AsaD029, AsaD031, AsaC249, and AsaD312 (Julian and Bartron 2007; Supplementary Table S.2). Fluorescently labeled PCR amplifications were pooled and simultaneously resolved via capillary electrophoresis by using a MegaBACE 1000 fluorescent genotyper (Amersham Biosciences, Piscataway, New Jersey). Allele sizes were determined in Fragment Profiler software (Amersham Biosciences) and were manually verified.

Population genetic analyses.—Microsatellite genotypes from each river sample were screened in the program MicroChecker (van Oosterhout et al. 2004) to test for evidence of null alleles, scoring errors, or large-allele dropout. To facilitate the conversion of microsatellite genotype data into file formats that were suitable for different population genetics software programs, we used the program CONVERT version 1.31 (Glaubitz 2004). Tests of genotypic linkage disequilibrium and departures from Hardy–Weinberg equilibrium (HWE) were performed in GENEPOP version 1.2 (Raymond and Rousset 1995) using the default Markov-chain parameters. Conformance to HWE was assessed for each locus as well as over all loci for each population by using exact tests, where the significance of tests across loci was determined with Fisher’s method. Observed heterozygosity (He), unbiased expected heterozygosity (He), and the effective number of alleles (Ae) were calculated in GenAIEx version 6.501 (Peakall and Smouse 2006, 2012) and were averaged over loci for each population. We estimated allelic richness (Arich) in the program HP-Rare, which uses the method of rarefaction to account for bias in estimates of Arich due to unequal sample sizes (Kalinowski 2005). The minimum number of genes for Arich estimates was set to 42. The Pam04 (n = 15) and Pot93 (n = 19) samples were omitted from rarefaction analyses because of their comparatively small sample sizes. Wilcoxon signed rank tests (Zar 1999) were used to test for significant changes in genetic diversity measures (Ae, He, Hr, and Arich) between pre- and post-supplementation samples. In rivers with multiple pre- and post-supplementation samples, collections were pooled and genetic diversity measures were re-calculated based on the pooled samples prior to statistical

| River      | 1992 | 1993 | 1994 | 1996 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | Total |
|------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|-------|
| James      | 32   | 37   | 38   | 31   | 76   | 34   | 147  | 87   | 83   | 565  |
| Pamunkey   | 39   | 95   | 91   | 64   | 54   | 32   | 53   | 15   | 30   | 122  | 39   | 168  |
| Rappahannock | 36   | 66   | 229  | 19   | 149  | 25   | 127  |
| Susquehanna | 90   | 57   | 87   | 83   | 144  |
| Nanticoke  | 28   | 28   | 546  |
| Patuxent   |      |      | 533  |

| Potomac | 36   | 66   | 129  | 144  |
| Rappahannock | 25   | 319  |
| Susquehanna | 229  | 319  |
| Nanticoke  | 87   | 144  |
| Patuxent   | 28   | 28   | 1,985|

Table 1. Rivers sampled, sample sizes, and years of American Shad collection from major Chesapeake Bay tributaries. Samples for some years are missing; however, the data are ordered chronologically.
testing. Just as was done for the individual river collection \( A_{rich} \) analyses, Pot1993 was not included in testing of \( A_{rich} \) due to its small sample size. The inbreeding coefficient \( F_{IS} \) was estimated for each locus in GenAlEx and then was averaged over loci.

Significant differences in allele frequency distribution between each pairwise grouping were assessed using genic contingency table tests in GENEPOP version 1.2 (Raymond and Rousset 1995). Exact \( P \)-values of these tests were calculated via a Markov-chain algorithm, and \( P \)-values were combined over loci by using Fisher’s method (Raymond and Rousset 1995). Although this test yields a \( P \)-value indicating significance, it provides little information about the magnitude of differentiation among collections, which can make the biological significance of the test somewhat difficult to interpret (Waples 1998). Therefore, we also investigated pairwise population differentiation by calculating the standardized differentiation index \( F_{ST} \) in GenAlEx version 6.501 for each pair of collections. The notation \( F_{ST}^{'} \) (as opposed to \( F_{ST} \)) denotes application of the scaling procedure described by Meirmans (2006), which ensures that \( F_{ST}^{'} \) can have a maximum value of 1.0 regardless of allelic variation within populations (Bird et al. 2011). The value \( F_{ST}^{'} \) is calculated within an analysis of molecular variance (AMOVA) framework and uses a pairwise, allele-by-allele distance matrix that accounts for intrapopulation variation as opposed to the genotypic distance matrix used by \( F_{ST} \). Calculated in this manner, \( F_{ST}^{'} \) is a useful index of population differentiation, indicating the extent to which populations share alleles. An \( F_{ST}^{'} \) value of zero equates to identical distribution of alleles, and an \( F_{ST}^{'} \) value of 1.0 equates to a completely nonoverlapping distribution of alleles (Bird et al. 2011). Significance of \( F_{ST}^{'} \) was assessed through 10,000 permutations.

Hierarchical AMOVAs applied to different groupings of the collections were performed in GenAlEx version 6.501. Values of \( F_{CT}^{'} \) symbolizing the partitioning of genetic variance due to differences among groups relative to the total genetic variance, where “C” denotes a chosen grouping of collections, were standardized to \( F_{CT}^{''} \) using the scaling procedure of Meirman (2006) implemented in GenAlEx. We compared the pre-supplementation James River versus Pamunkey River samples as well as the post-supplementation James River versus Pamunkey River samples (within-river collections were pooled and treated as groups; \( F_{CT}^{''} \)) to examine the extent to which supplementation altered genetic differentiation between populations in these two systems. Similar analyses were applied to the pre- versus post-supplementation James River collections and the pre- versus post-supplementation Pamunkey River collections (\( F_{CT}^{'''} \)) to characterize genetic changes within each of these two populations. To characterize the wider Chesapeake Bay, we analyzed pre- and post-supplementation Chesapeake Bay-wide collections treated as groups (\( F_{CT}^{''''} \)), with temporal samples pooled within rivers. Pre-supplementation Chesapeake Bay consisted of all pre-supplementation samples and early Susquehanna River samples. Post-supplementation Chesapeake Bay included all contemporary samples except the James and Pamunkey River samples collected prior to 2006. Significance of each \( F_{CT}^{'''} \) value was assessed by 10,000 permutations.

A priori assignment of American Shad collections by river of capture for analyses of population structure may fail to accurately describe the true genetic relationships. For example, most Chesapeake Bay tributaries contain mixtures of more than one population because of intentional stocking with extrabasin fish (Supplementary Table S.1), so the treatment of yearly samples from these rivers as single populations may not be appropriate. To investigate this possibility, we used the program STRUCTURE version 2.3.1 (Pritchard et al. 2000) to complement traditional methods of analyzing population structure (e.g., AMOVA and pairwise tests of genic differentiation) that require a priori grouping of populations. STRUCTURE places individuals into clusters in a manner that minimizes linkage disequilibrium and maximizes HWE expectations within clusters. The admixture model and correlated allele frequencies options were selected for STRUCTURE simulations because our American Shad data set exhibited low levels of differentiation and was affected by extensive stocking history. We analyzed the baseline populations (Rap92 and Rap93; Sus92; Jam92 and Jam93; Pam92, Pam93, Pam94, and Pam96; Pot 93; and Nan93) at 1–6 clusters (\( K = \) number of clusters) to evaluate whether population structure was detectable among Chesapeake Bay tributaries prior to extensive supplementation of the James River population as well as other Chesapeake Bay populations. Another analysis for post-supplementation collections from these same populations (and including Pat07) was performed.

An additional STRUCTURE analysis was performed using only the James and Pamunkey River populations because although low levels of population structure among Chesapeake Bay tributaries suggest that migration may be high due to natural (straying) and human (supplementation) factors, the OTC data indicate that there are very few strays present on the spawning grounds in the James River or Pamunkey River (VDGIF 2009). An overwhelming majority of our American Shad samples from James and Pamunkey River spawning grounds already were known to arise from only these two rivers. In addition, we hypothesized that we might garner increased sensitivity to detect population structure between James and Pamunkey River American Shad if we omitted other populations that were not as likely to have contributed. All simulations were set to discard the first 100,000 iterations as burn-in and were run for an additional 200,000 iterations. Trace plots of the admixture parameter \( \alpha \), likelihood of the data, and the estimate of the posterior probability \( \ln(P|D) \) were visually inspected for convergence of chains. Each run for each number of clusters was iterated three times to evaluate consistency across runs. Population structure bar plots were created using DISTRUPT (Rosenberg 2004). We chose the most biologically sensible clustering solution by following the guidelines in the STRUCTURE manual (Pritchard et al. 2007) in conjunction with the \( \Delta K \) criterion of Evanno et al. (2005).
All statistical tests were evaluated at an α value of 0.05; in cases of multiple independent statistical tests, we employed a sequential Bonferroni correction to control for the increased chance of type I error (Rice 1989).

RESULTS

Null Alleles

The presence of null alleles was suggested by MicroChecker at least once at each locus in the 30 river samples. These occurrences appeared largely random among populations and loci, with the exception of Pam00, Pam01, and Pam02, each of which was implicated as having null alleles at Asa-8, AsaB20, and Asa-9. No other samples or loci shared this pattern, and therefore the prediction of null alleles at these loci and population samples was not supported by possible PCR or genotyping artifacts. There was no indication of large-allele dropout or systematic scoring error, so all loci were retained for further analyses.

Hardy–Weinberg Equilibrium

Multilocus tests of HWE using Fisher’s method indicated that 9 of 30 collections deviated significantly from Hardy–Weinberg proportions after sequential Bonferroni correction (Table 2). Subsequent one-sided U-tests for heterozygote deficiency in GENEPOP were significant for these same nine collections (data not shown). However, of the 270 tests conducted to evaluate conformity to HWE for individual loci in each collection, only seven remained significant after sequential Bonferroni correction.

| River       | Year | n   | $H_e$ | $H_o$ | $A_e$ | $A_{rich}$ | $F_{IS}$ | HWE  |
|-------------|------|-----|-------|-------|-------|------------|----------|------|
| James       | 1992 | 32  | 0.82  | 0.74  | 5.89  | 9.43       | 0.08     | <0.001|
|             | 1993 | 37  | 0.82  | 0.78  | 6.13  | 9.45       | 0.03     | 0.615 |
|             | 2000 | 38  | 0.83  | 0.77  | 6.09  | 9.33       | 0.07     | 0.043 |
|             | 2002 | 31  | 0.81  | 0.73  | 5.42  | 9.35       | 0.09     | 0.130 |
|             | 2004 | 76  | 0.82  | 0.76  | 6.27  | 9.70       | 0.06     | 0.145 |
|             | 2006 | 34  | 0.82  | 0.78  | 5.82  | 9.49       | 0.03     | 0.062 |
|             | 2007 | 147 | 0.82  | 0.77  | 6.14  | 9.39       | 0.05     | 0.496 |
|             | 2008 | 87  | 0.82  | 0.79  | 5.99  | 9.66       | 0.03     | <0.001|
|             | 2009 | 83  | 0.82  | 0.73  | 6.24  | 9.62       | 0.11     | <0.001|
| Pamunkey    | 1992 | 39  | 0.81  | 0.81  | 6.36  | 9.46       | -0.01    | 0.320 |
|             | 1993 | 95  | 0.82  | 0.79  | 6.23  | 9.54       | 0.03     | 0.302 |
|             | 1994 | 91  | 0.82  | 0.77  | 6.08  | 9.70       | 0.05     | 0.485 |
|             | 1996 | 64  | 0.82  | 0.80  | 6.25  | 9.42       | 0.02     | 0.526 |
|             | 2000 | 54  | 0.81  | 0.73  | 6.16  | 9.44       | 0.09     | <0.001|
|             | 2001 | 32  | 0.81  | 0.67  | 5.45  | 8.21       | 0.17     | <0.001|
|             | 2002 | 53  | 0.82  | 0.71  | 6.06  | 9.43       | 0.12     | <0.001|
|             | 2004 | 15  | 0.82  | 0.82  | 5.10  |           | -0.04    | 0.507 |
|             | 2005 | 30  | 0.81  | 0.78  | 5.64  | 9.10       | 0.02     | 0.009 |
|             | 2007 | 122 | 0.82  | 0.74  | 6.36  | 9.48       | 0.10     | <0.001|
|             | 2008 | 39  | 0.80  | 0.69  | 5.42  | 8.86       | 0.12     | <0.001|
| Rappahannock| 1992 | 36  | 0.82  | 0.84  | 6.33  | 9.39       | -0.04    | 0.927 |
|             | 1993 | 66  | 0.80  | 0.77  | 5.92  | 9.22       | 0.04     | 0.365 |
|             | 2008 | 25  | 0.80  | 0.81  | 5.49  |           | -0.03    | 0.270 |
| Susquehanna | 1992 | 90  | 0.76  | 0.73  | 4.66  | 8.27       | 0.04     | 0.030 |
|             | 2007 | 229 | 0.82  | 0.79  | 6.53  | 9.57       | 0.03     | 0.057 |
| Nanticoke   | 1993 | 57  | 0.80  | 0.77  | 5.79  | 9.62       | 0.03     | 0.006 |
|             | 2007 | 87  | 0.81  | 0.80  | 5.89  | 9.39       | 0.01     | 0.069 |
| Potomac     | 1993 | 19  | 0.81  | 0.82  | 5.27  |           | -0.05    | 0.027 |
|             | 2007 | 149 | 0.82  | 0.71  | 6.44  | 9.43       | 0.12     | <0.001|
| Patuxent    | 2007 | 28  | 0.79  | 0.74  | 5.30  | 8.89       | 0.05     | 0.080 |
| Total       |      | 1,985 | | | | | |
Genetic Diversity

All Chesapeake Bay populations exhibited relatively high levels of genetic variation that were comparable to those observed in other studies of Chesapeake Bay American Shad (Waters et al. 2000; Hasselman et al. 2013). Values of $H_e$ ranged from 0.67 (Pam01) to 0.84 (Rap92); $H_r$ ranged from 0.76 (Sus92) to 0.83 (Jam00); and $A_{rich}$ estimates ranged from 8.21 (Pam01) to 9.70 (Pam94 and Jam04; Table 2). Values of $A_e$ ranged from 4.66 (Sus92) to 6.53 (Sus07). Levels of $H_e$, $H_r$, $A_{rich}$, and $A_e$ were similar and not significantly different between pooled pre- and post-supplementation samples collected in the James, Nanticoke, and Rappahannock rivers (Supplementary Table S.3). However, $H_e$ declined significantly from 0.79 to 0.72 between pooled pre- and post-supplementation Potomac River samples ($P = 0.018$) and from 0.82 to 0.71 between pre- and post-supplementation Potomac River samples ($P = 0.007$). All four genetic diversity measures declined significantly during the period for Susquehanna River collections (all $P < 0.03$). The $F_{IS}$ estimates ranged from $-0.05$ (Pot93) to 0.17 (Pam01), suggesting that levels of inbreeding were not excessive.

Genetic Differentiation and Population Structure

Pairwise tests of genic differentiation (Supplementary Table S.4) revealed that there were no significant differences among collections within rivers with multiple pre-supplementation samples (James, Pamunkey, and Rappahannock rivers). Therefore, over these short time spans, the collections appeared to be temporally stable. The same result was obtained for post-supplementation collections with temporal samples (i.e., 2006 and onward from the James and Pamunkey rivers). Within rivers, the Jam93 sample was notable for having five significant comparisons with later collections from the James River. Comparison of the pre-supplementation James and Pamunkey River samples indicated no significant differences between Jam92 and any of the pre-supplementation Pamunkey River samples, yet Jam93 was significantly different from Pam93, Pam94, and Pam96. The Sus92 sample exhibited significant differentiation from Sus07 and all other collections. Among post-supplementation samples collected during the same year, significant differences were observed between the following pairs: Nan07 and Jam07; Jam07 and Sus07; Pam07 and Sus07; and Nan07 and Pot07. Other significant differences tended to occur between rivers (e.g., Sus07 and Jam93) or within rivers (e.g., Pam01 and Pam08).

Pairwise differentiation calculated as $F'_{ST}$ was low and nonsignificant between most pairs of collections (Table 3); 49% of pairwise comparisons resulted in $F'_{ST}$ of 0.01 or less. Significant values of $F'_{ST}$ (range = 0.09–0.23; $P < 0.001$) were only observed in comparisons of Sus92 with all other collections and between Pam02 and Rap08. Only the Susquehanna River population showed evidence of significant temporal differentiation (Sus92 and Sus07; $F'_{ST} = 0.1531; P < 0.001$). Within the James River, Jam93 showed higher pairwise $F'_{ST}$ ($F'_{ST}$ range = 0.003–0.027) than Jam92 ($F'_{ST}$ range = 0.034–0.050) in comparisons with post-supplementation James River collections; this finding was similar to the genetic tests of differentiation, although none of these $F'_{ST}$ comparisons was significant. The pre- and post-supplementation Nanticoke, Rappahannock, and Potomac River populations exhibited low levels of differentiation ($F'_{ST} \leq 0.069; P > 0.05$) and were not significantly different from each other. The sample from the Patuxent River, which had no pre-supplementation complement, exhibited low levels of differentiation from all other collections ($F'_{ST} \leq 0.043; P > 0.05$) except Sus92.

Pre-supplementation James and Pamunkey River collections that were treated as separate groups for hierarchical AMOVAs (Table 4) exhibited a low but significant level of differentiation ($F_{CT}' = 0.012; P = 0.017$), suggesting that they were subtly different populations. In contrast, the post-supplementation James and Pamunkey River collections showed a reduced level of differentiation ($F_{CT}' = 0.007; P = 0.029$). Analysis of pre- versus post-supplementation James River samples produced an $F_{CT}'$ value of 0.032 ($P < 0.001$), indicating that pre-supplementation James River American Shad were different from the post-supplementation James River population. In contrast, differentiation was much lower between the pre- and post-supplementation Pamunkey River samples ($F_{CT}' = 0.007; P = 0.038$). Collectively, these results suggest that the Pamunkey River American Shad population has remained relatively unchanged during the period of supplementation, whereas the James River population has become more similar to the Pamunkey River population. Within the broader Chesapeake Bay, differentiation was greater among pre-supplementation collections ($F_{CT}' = 0.066; P < 0.001$) than among post-supplementation collections ($F_{CT}' = 0.004; P = 0.106$). Examination of pre- versus post-supplementation Chesapeake Bay collections ($F_{CT}' = 0.067; P = 0.879$) indicated that genetic
| Collection | Jam92 | Jam93 | Jam00 | Jam02 | Jam04 | Jam06 | Jam07 | Jam08 | Jam09 |
|------------|------|------|------|------|------|------|------|------|------|
| Jam92      | —    | 0.3553 | 0.4615 | 0.0260 | 0.4624 | 0.4011 | 0.0154 | 0.0688 | 0.0470 |
| Jam93      | 0.0043 | —    | 0.0564 | 0.0540 | 0.0217 | 0.0226 | 0.0011 | 0.0034  | 0.0002 |
| Jam00      | 0.0000 | 0.0251 | —    | 0.4648 | 0.4594 | 0.1849 | 0.4564 | 0.4579 | 0.3490 |
| Jam02      | 0.0367 | 0.0267 | 0.0000 | —    | 0.2118 | 0.4253 | 0.4628 | 0.2480 | 0.4581 |
| Jam04      | 0.0000 | 0.0253 | 0.0000 | 0.0089 | —    | 0.4527 | 0.0636 | 0.1927 | 0.0213 |
| Jam06      | 0.0025 | 0.0367 | 0.0132 | 0.0013 | 0.0000 | —    | 0.2402 | 0.4576 | 0.4572 |
| Jam07      | 0.0274 | 0.0402 | 0.0000 | 0.0000 | 0.0088 | 0.0062 | —    | 0.0969 | 0.1295 |
| Jam08      | 0.0184 | 0.0336 | 0.0000 | 0.0066 | 0.0054 | 0.0000 | 0.0066 | —    | 0.1874 |
| Jam09      | 0.0218 | 0.0501 | 0.0032 | 0.0000 | 0.0160 | 0.0000 | 0.0000 | 0.0059 | 0.0559 |
| Pam92      | 0.0000 | 0.0310 | 0.0166 | 0.0312 | 0.0000 | 0.0000 | 0.0154 | 0.0108 | 0.0163 |
| Pam93      | 0.0014 | 0.0101 | 0.0000 | 0.0023 | 0.0009 | 0.0023 | 0.0012 | 0.0006 | 0.0101 |
| Pam94      | 0.0184 | 0.0202 | 0.0048 | 0.0099 | 0.0042 | 0.0090 | 0.0000 | 0.0028 | 0.0120 |
| Pam96      | 0.0000 | 0.0203 | 0.0000 | 0.0000 | 0.0000 | 0.0010 | 0.0000 | 0.0000 | 0.0000 |
| Pam00      | 0.0000 | 0.0217 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0061 |
| Pam01      | 0.0171 | 0.0570 | 0.0000 | 0.0152 | 0.0041 | 0.0433 | 0.0119 | 0.0158 | 0.0117 |
| Pam02      | 0.0289 | 0.0504 | 0.0120 | 0.0074 | 0.0444 | 0.0000 | 0.0256 | 0.0129 | 0.0031 |
| Pam04      | 0.0000 | 0.0199 | 0.0064 | 0.0000 | 0.0000 | 0.0318 | 0.0049 | 0.0166 | 0.0117 |
| Pam05      | 0.0309 | 0.0356 | 0.0000 | 0.0000 | 0.0132 | 0.0359 | 0.0000 | 0.0000 | 0.0000 |
| Pam07      | 0.0145 | 0.0159 | 0.0033 | 0.0000 | 0.0000 | 0.0108 | 0.0114 | 0.0000 | 0.0167 |
| Pam08      | 0.0051 | 0.0265 | 0.0000 | 0.0000 | 0.0132 | 0.0116 | 0.0011 | 0.0018 | 0.0102 |
| Rap92      | 0.0246 | 0.0413 | 0.0000 | 0.0000 | 0.0000 | 0.0040 | 0.0000 | 0.0086 | 0.0000 |
| Rap93      | 0.0072 | 0.0354 | 0.0100 | 0.0386 | 0.0140 | 0.0073 | 0.0196 | 0.0144 | 0.0245 |
| Rap08      | 0.0524 | 0.0526 | 0.0619 | 0.0690 | 0.0516 | 0.0609 | 0.0483 | 0.0575 | 0.0569 |
| Sus92      | **0.2000** | **0.2290** | **0.1576** | **0.1543** | **0.1570** | **0.1587** | **0.1343** | **0.1671** | **0.1400** |
| Sus07      | 0.0159 | 0.0364 | 0.0119 | 0.0142 | 0.0036 | 0.0136 | 0.0222 | 0.0121 | 0.0177 |
| Nan93      | 0.0164 | 0.0337 | 0.0076 | 0.0092 | 0.0020 | 0.0021 | 0.0046 | 0.0027 | 0.0151 |
| Nan07      | 0.0048 | 0.0331 | 0.0112 | 0.0121 | 0.0148 | 0.0089 | 0.0142 | 0.0126 | 0.0097 |
| Pot93      | 0.0270 | 0.0651 | 0.0269 | 0.0400 | 0.0218 | 0.0434 | 0.0280 | 0.0360 | 0.0202 |
| Pot07      | 0.0089 | 0.0312 | 0.0042 | 0.0124 | 0.0045 | 0.0153 | 0.0061 | 0.0071 | 0.0155 |
| Pat07      | 0.0272 | 0.0416 | 0.0139 | 0.0000 | 0.0029 | 0.0000 | 0.0039 | 0.0069 | 0.0000 |

**TABLE 3.** Pairwise matrix of $F_{ST}$ values (below diagonal) and $P$-values (above diagonal) for Chesapeake Bay populations of American Shad. Negative $F_{ST}$ values were converted to zero. Collection codes indicate river (Jam = James River; Pam = Pamunkey River; Rap = Rappahannock River; Sus = Susquehanna River; Nan = Nanticoke River; Pot = Potomac River; Pat = Patuxent River) and year of sampling. Bold italics indicate statistically significant comparisons.
### TABLE 3. Extended.

| Collection | Pam92  | Pam93  | Pam94  | Pam96  | Pam00  | Pam01  | Pam02  | Pam04  | Pam05  | Pam07  | Pam08  |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Jam92      | 0.466  | 0.4178 | 0.0682 | 0.4650 | 0.4515 | 0.1562 | 0.0319 | 0.4576 | 0.0539 | 0.0920 | 0.3326 |
| Jam93      | 0.0275 | 0.1550 | 0.0419 | 0.0537 | 0.0521 | 0.0020 | 0.0014 | 0.2012 | 0.0280 | 0.0541 | 0.0475 |
| Jam00      | 0.1273 | 0.4525 | 0.2908 | 0.4662 | 0.4561 | 0.4573 | 0.1602 | 0.3693 | 0.4652 | 0.3314 | 0.4642 |
| Jam02      | 0.0531 | 0.3815 | 0.1764 | 0.4636 | 0.4579 | 0.1643 | 0.2646 | 0.4610 | 0.4567 | 0.4587 | 0.4698 |
| Jam04      | 0.4547 | 0.3992 | 0.2363 | 0.4572 | 0.4561 | 0.3301 | 0.2739 | 0.4575 | 0.1376 | 0.4530 | 0.1035 |
| Jam06      | 0.4628 | 0.3761 | 0.1931 | 0.4239 | 0.4605 | 0.0121 | 0.4580 | 0.1206 | 0.3235 | 0.1370 | 0.1955 |
| Jam07      | 0.0531 | 0.3710 | 0.4676 | 0.4686 | 0.4676 | 0.1224 | 0.0022 | 0.3686 | 0.4670 | 0.0117 | 0.4088 |
| Jam08      | 0.1399 | 0.4209 | 0.2851 | 0.4564 | 0.3830 | 0.0920 | 0.0691 | 0.1950 | 0.4611 | 0.4690 | 0.3868 |
| Jam09      | 0.0677 | 0.0629 | 0.0402 | 0.4508 | 0.2056 | 0.1552 | 0.3319 | 0.2710 | 0.4631 | 0.0060 | 0.1502 |
| Pam92      | —      | 0.0476 | 0.0368 | 0.2300 | 0.2931 | 0.0940 | 0.1845 | 0.0682 | 0.1274 | 0.1570 | 0.0397 |
| Pam93      | 0.0175 | —      | 0.4522 | 0.4663 | 0.4531 | 0.3390 | 0.0166 | 0.4477 | 0.4233 | 0.4092 | 0.4158 |
| Pam94      | 0.0198 | 0.0000 | —      | 0.4607 | 0.4613 | 0.1019 | 0.0031 | 0.4465 | 0.2587 | 0.1235 | 0.4527 |
| Pam96      | 0.0077 | 0.0000 | 0.0000 | —      | 0.4594 | 0.1966 | 0.1819 | 0.3796 | 0.3856 | 0.4623 | 0.4642 |
| Pam00      | 0.0055 | 0.0003 | 0.0000 | 0.0000 | —      | 0.4507 | 0.0320 | 0.4520 | 0.4606 | 0.4625 | 0.4555 |
| Pam01      | 0.0205 | 0.0038 | 0.0148 | 0.0103 | 0.0000 | —      | 0.0993 | 0.4170 | 0.3785 | 0.1761 | 0.0579 |
| Pam02      | 0.0103 | 0.0200 | 0.0282 | 0.0082 | 0.0209 | 0.0178 | —      | 0.2798 | 0.3078 | 0.1073 | 0.0146 |
| Pam04      | 0.0402 | 0.0000 | 0.0006 | 0.0050 | 0.0000 | 0.0029 | 0.0118 | —      | 0.3392 | 0.4584 | 0.1557 |
| Pam05      | 0.0180 | 0.0011 | 0.0066 | 0.0024 | 0.0000 | 0.0034 | 0.0057 | 0.0082 | —      | 0.3261 | 0.4566 |
| Pam07      | 0.0088 | 0.0006 | 0.0059 | 0.0000 | 0.0000 | 0.0095 | 0.0091 | 0.0000 | 0.0000 | 0.0025 | 0.0002 |
| Pam08      | 0.0262 | 0.0011 | 0.0000 | 0.0000 | 0.0000 | 0.0255 | 0.0304 | 0.0250 | 0.0000 | 0.0158 | —      |
| Rap92      | 0.0028 | 0.0052 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0025 | 0.0002 |
| Rap93      | 0.0210 | 0.0015 | 0.0114 | 0.0127 | 0.0042 | 0.0126 | 0.0417 | 0.0370 | 0.0233 | 0.0189 | 0.0060 |
| Rap08      | 0.0444 | 0.0442 | 0.0288 | 0.0590 | 0.0657 | 0.0705 | 0.0994 | 0.0550 | 0.0491 | 0.0356 | 0.0590 |
| Sus92      | 0.1495 | 0.1687 | 0.1328 | 0.1540 | 0.1445 | 0.1775 | 0.1606 | 0.1949 | 0.1659 | 0.1629 | 0.1343 |
| Sus07      | 0.0039 | 0.0086 | 0.0066 | 0.0087 | 0.0000 | 0.0076 | 0.0169 | 0.0058 | 0.0000 | 0.0156 | 0.0112 |
| Nan93      | 0.0065 | 0.0005 | 0.0064 | 0.0045 | 0.0043 | 0.0248 | 0.0275 | 0.0284 | 0.0037 | 0.0078 | 0.0000 |
| Nan07      | 0.0257 | 0.0000 | 0.0140 | 0.0063 | 0.0009 | 0.0131 | 0.0202 | 0.0303 | 0.0236 | 0.0175 | 0.0054 |
| Pot93      | 0.0334 | 0.0216 | 0.0190 | 0.0353 | 0.0424 | 0.0249 | 0.0193 | 0.0000 | 0.0581 | 0.0030 | 0.0546 |
| Pot07      | 0.0075 | 0.0090 | 0.0011 | 0.0000 | 0.0000 | 0.0159 | 0.0109 | 0.0000 | 0.0000 | 0.0084 | 0.0000 |
| Pat07      | 0.0125 | 0.0149 | 0.0057 | 0.0000 | 0.0018 | 0.0270 | 0.0024 | 0.0380 | 0.0000 | 0.0082 | 0.0034 |
### TABLE 3. Extended.

| Collection | Rap92 | Rap93 | Rap08 | Sus92 | Sus07 | Nan93 | Nan07 | Pot93 | Pot07 | Pat07 |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Jam92      | 0.0751| 0.2618| 0.0128| 0.0001| 0.0561| 0.1073| 0.3053| 0.1286| 0.1881| 0.0734|
| Jam93      | 0.0100| 0.0068| 0.0118| 0.0001| 0.0008| 0.0085| 0.0071| 0.0060| 0.0037| 0.0145|
| Jam00      | 0.4631| 0.1779| 0.0028| 0.0001| 0.0895| 0.2337| 0.1392| 0.1038| 0.2928| 0.1800|
| Jam02      | 0.4637| 0.0062| 0.0020| 0.0001| 0.0794| 0.2118| 0.1347| 0.0467| 0.1157| 0.4590|
| Jam04      | 0.4674| 0.0443| 0.0022| 0.0001| 0.2066| 0.3577| 0.0245| 0.1133| 0.1892| 0.3681|
| Jam06      | 0.3584| 0.2469| 0.0047| 0.0001| 0.0766| 0.3892| 0.1933| 0.0441| 0.0798| 0.4541|
| Jam07      | 0.4582| 0.0041| 0.0021| 0.0001| 0.0005| 0.2166| 0.0101| 0.0554| 0.0614| 0.3264|
| Jam08      | 0.1954| 0.0320| 0.0003| 0.0001| 0.0088| 0.3242| 0.0338| 0.0310| 0.0757| 0.2606|
| Jam09      | 0.4560| 0.0039| 0.0010| 0.0001| 0.0012| 0.0371| 0.0726| 0.1284| 0.0061| 0.4642|
| Pam92      | 0.3825| 0.0414| 0.0140| 0.0001| 0.2863| 0.2422| 0.0142| 0.0712| 0.1778| 0.2016|
| Pam93      | 0.2794| 0.3787| 0.0039| 0.0001| 0.0312| 0.4307| 0.4594| 0.1112| 0.0446| 0.1094|
| Pam94      | 0.4676| 0.0656| 0.0301| 0.0001| 0.0720| 0.1805| 0.0228| 0.1285| 0.3685| 0.2909|
| Pam96      | 0.4585| 0.0746| 0.0013| 0.0001| 0.0639| 0.2765| 0.1816| 0.0392| 0.4545| 0.4606|
| Pam00      | 0.4384| 0.2828| 0.0005| 0.0001| 0.4535| 0.2811| 0.4188| 0.0221| 0.4707| 0.4082|
| Pam01      | 0.4569| 0.1462| 0.0013| 0.0001| 0.1935| 0.0353| 0.1206| 0.1346| 0.0750| 0.0662|
| Pam02      | 0.4681| 0.0004| 0.0001| 0.0001| 0.0112| 0.0079| 0.0150| 0.1568| 0.0678| 0.3904|
| Pam04      | 0.4666| 0.0620| 0.0417| 0.0001| 0.3467| 0.1011| 0.0838| 0.4506| 0.4602| 0.0865|
| Pam05      | 0.4650| 0.0515| 0.0165| 0.0001| 0.4589| 0.3561| 0.0375| 0.0168| 0.4558| 0.4547|
| Pam07      | 0.3711| 0.0059| 0.0097| 0.0001| 0.0003| 0.1184| 0.0034| 0.3818| 0.0327| 0.2101|
| Pam08      | 0.4485| 0.2549| 0.0036| 0.0001| 0.0820| 0.4549| 0.2578| 0.0157| 0.4575| 0.3773|
| Rap92      | —     | 0.0120| 0.0045| 0.0001| 0.4548| 0.2695| 0.1496| 0.1395| 0.4650| 0.4486|
| Rap93      | 0.0313| —     | 0.0006| 0.0001| 0.0023| 0.4562| 0.0675| 0.0049| 0.0011| 0.1734|
| Rap08      | 0.0590| 0.0643| —     | 0.0001| 0.0007| 0.0057| 0.0002| 0.1250| 0.0003| 0.0256|
| Sus92      | 0.1287| 0.1929| 0.1959| —     | 0.0001| 0.0001| 0.0001| 0.0001| 0.0001| 0.0001|
| Sus07      | 0.0000| 0.0198| 0.0495| 0.1531| —     | 0.0299| 0.0085| 0.0114| 0.0364| 0.4303|
| Nan93      | 0.0060| 0.0000| 0.0466| 0.1713| 0.0118| —     | 0.0695| 0.0192| 0.3749| 0.4623|
| Nan07      | 0.0106| 0.0113| 0.0699| 0.1657| 0.0122| 0.0116| —     | 0.0256| 0.0014| 0.2731|
| Pot93      | 0.0234| 0.0572| 0.0295| 0.1736| 0.0386| 0.0447| 0.0372| —     | 0.0230| 0.1249|
| Pot07      | 0.0000| 0.0241| 0.0558| 0.1555| 0.0059| 0.0013| 0.0189| 0.0348| —     | 0.1979|
| Pat07      | 0.0000| 0.0117| 0.0433| 0.1321| 0.0008| 0.0000| 0.0064| 0.0270| 0.0090| —     |
structure has changed appreciably since the 1990s among the Chesapeake Bay tributary populations we examined. When this result is further considered in light of the low level of differentiation observed in post-supplementation Chesapeake Bay, it suggests that Chesapeake Bay populations have become more similar to each other.

Levels of differentiation characterized by hierarchical AMOVA for the pre-supplementation Chesapeake Bay populations were largely corroborated by STRUCTURE analysis, which suggested the presence of two clusters: the early Susquehanna River comprised one cluster, and the other Chesapeake Bay tributary collections formed the second cluster (Figure 3A). Although the ΔK analysis suggested a K-value of 3, this appears to be an artifact of the decrease in average ln(K) at K-values greater than 2 (Supplementary Figure S.1A). In the analysis of post-supplementation Chesapeake Bay populations, including the James and Pamunkey River populations, a K-value of 1 was the solution with the highest probability and lowest variance, revealing no evidence of contemporary population substructure (Figure 3B). As was observed for the pre-supplementation Chesapeake Bay STRUCTURE analysis, the ΔK method suggested K-values of 3 and 5; again, this appeared to be an artifact of the rapid decrease in average ln(K) at K-values greater than 1 (Supplementary Figure S.1B). STRUCTURE analyses limited to only the pre-supplementation James and Pamunkey River collections of American Shad from Chesapeake tributaries before and after supplemental stocking (for all results, the source of variation = among groups).

| Comparison                        | df | $F_{CT}$ | P       |
|-----------------------------------|----|----------|---------|
| James River, pre- vs. post-supplementation | 1  | 0.032    | <0.001  |
| Pamunkey River, pre- vs. post-supplementation | 1  | 0.007    | 0.038   |
| James River, pre-supplementation vs. Pamunkey River, pre-supplementation | 1  | 0.013    | 0.017   |
| James River, post-supplementation vs. Pamunkey River, post-supplementation | 1  | 0.007    | 0.029   |
| Chesapeake Bay, pre-supplementation | 5  | 0.063    | <0.001  |
| Chesapeake Bay, post-supplementation | 6  | 0.004    | 0.106   |
| Chesapeake Bay, pre- vs. post-supplementation | 1  | 0.067    | 0.879   |

**FIGURE 3.** Bar plots for two clusters ($K = 2$) from STRUCTURE (Pritchard et al. 2000) analysis of Chesapeake Bay American Shad samples collected during (A) the pre-supplementation period, 1992–1996; and (B) the post-supplementation period, 2007–2008. The actual number of clusters for the post-supplementation Chesapeake Bay samples was $K = 1$, but results for $K = 2$ are shown for visual comparison with the pre-supplementation samples. Each individual American Shad is represented by a single vertical line, and the percent membership to each cluster (vertical axis) is illustrated by the two different colors. Populations are separated by vertical black lines.
lections and post-supplementation James and Pamunkey River collections revealed no evidence of population structure (data not shown).

**DISCUSSION**

The levels of genetic diversity we observed within the James River population, the Pamunkey River population, and all of the Chesapeake Bay populations (both pre-supplementation and contemporary samples) were consistent with those described by Hasselman et al. (2013), who sampled American Shad that were collected in 2003–2006 from coastal rivers throughout the species’ entire native range, including Chesapeake Bay. This result is similar to some analyses of genetic diversity in salmonids subjected to supportive breeding programs, where high levels of diversity in the recipient populations persist after years of intensive stocking (Heggenes et al. 2006; Eldridge and Kilberg 2008). It is notable, however, that while the magnitude of the change was not great, genetic diversity appears to have declined in the Susquehanna River population, and continued genetic monitoring should be implemented to monitor this trend. In James River American Shad, retention of genetic diversity in relation to VDGIF hatchery practices was investigated by Brown et al. (2000), who found that although there was significant reproductive variance in the hatchery, the larvae that were stocked into the James River tended to fully represent the genetic diversity of their parents through the point of stocking. Data from the current study reinforce this conclusion and imply that diversity is preserved through the adult stage as well. In the Pamunkey River population, the significant decrease in \( H_s \) suggests that genetic diversity may be declining, but similar decreases were not observed for \( A_{rich}, A_e, \) or \( H_e \). Nevertheless, given the current usage of the Pamunkey River as a source of broodstock for the James River, continued monitoring of trends in genetic diversity for this population would be beneficial.

Our data for pre-supplementation American Shad in the James and Pamunkey rivers are consistent with the work of Waters et al. (2000) and Brown et al. (2000), who concluded that pre-supplementation populations of American Shad from the two rivers exhibited subtle genetic differentiation. However, among the six Chesapeake Bay American Shad populations that were sampled in the early 1990s, only the Susquehanna River population was strongly differentiated from the others. This is reasonable given that although all Chesapeake Bay tributaries were stocked between the late 1800s and the 1940s (Mansueti and Kolb 1953), heavy and consistent supplementation of the Susquehanna River population was initiated again in the 1970s (Hendricks 2003; St. Pierre 2003), whereas other Chesapeake Bay rivers experienced a respite from stocking until the 1990s (Supplementary Table S.1). We hypothesize that high reproductive variance in the hatchery, reproductive variance among naturally spawning adults, differential recruitment of small numbers of stocked American Shad, or a combination of these factors contributed to genetic drift in and resultant differentiation of the early Susquehanna River population from the other Chesapeake Bay populations sampled in 1992–1993.

The contemporary Susquehanna River American Shad population was found to be similar to other extant Chesapeake Bay populations and significantly different from its 1990s genetic complement. Hasselman et al. (2013) also documented that the contemporary Susquehanna River population was similar to other contemporary Chesapeake Bay samples. This increase in similarity to populations in other Chesapeake Bay tributaries may have resulted from effective migration via straying from other Chesapeake Bay tributaries and increased hatchery recruitment in the latter years of the Susquehanna River restoration effort. High straying rates for American Shad in the Pamunkey River have been documented using otolith chemical signatures (Walther 2008), suggesting that straying could be high among other river systems as well. In a genetic context, a straying rate of 1% in large American Shad populations could mean hundreds of breeding immigrants per generation (Waters et al. 2000). With regard to recruitment of hatchery-produced American Shad, after 1992 the number of American Shad returning to Conowingo Dam increased dramatically, with over 200,000 American Shad passing through the Conowingo Dam fish lifts in 2003 (St. Pierre 2003), many of which were of Chesapeake Bay, Delaware River, and Hudson River origin. These populations all exhibited relatively low levels of among-population genetic differentiation (Brown et al. 1999; Waters et al. 2000; Hasselman et al. 2013) when analyzed with pairwise \( F_{ST} \), Bayesian clustering, and hierarchical AMOVA.

Genetic data for pre- versus post-supplementation James and Pamunkey River American Shad populations (a 17-year period) indicated that any prior genetic differentiation has dissipated. Furthermore, contemporary samples from the seven major Chesapeake Bay populations showed nonsignificant among-population genetic differentiation. Our results are in agreement with those from Hasselman et al. (2013) who found comparable low levels of differentiation among several Chesapeake Bay populations. It is possible that the lack of differentiation among contemporary American Shad populations in Chesapeake Bay relative to the levels of differentiation we observed for the 1990s is due to the extensive supplementation occurring over nearly two decades. Unfortunately, comparative samples of American Shad collected prior to the 1990s do not exist. Assessment of true baseline differentiation among Chesapeake Bay American Shad would require samples from the early 1800s, prior to the rampant supplementation that took place throughout the basin from the late 1800s to mid-1900s. Regardless, our temporal sampling (albeit brief) provides a window into how population structure in Chesapeake Bay American Shad has changed in the presence of supplementation.

Regarding the differentiation detected between the pre-supplementation James and Pamunkey River populations, it is reasonable to ask whether these subtle differences were biologically meaningful and whether the lack of differentiation between the contemporary James and Pamunkey River populations
heralds the loss of unique adaptive variation. Conservation genetic studies tend to view the patterns of genetic variation observed with neutral markers as a proxy for adaptive variability among populations (Reed and Frankham 2001); that is, high heterozygosity at microsatellites is correlated with high levels of quantitative trait variation, and low levels of neutral differentiation likely mean little difference in adaptive variability. However, neutral markers are poor predictors of adaptive genetic differences among populations (McKay and Latta 2002). To date, there has been no examination of quantitative trait loci in American Shad for the purposes of examining interpopulation differences. Future studies of American Shad population structure utilizing traits under selection may provide some insight into whether populations have unique adaptive potential. For example, studies of the major histocompatibility complex in Pacific salmon populations have reported increased resolution of genetically similar and geographically proximal populations relative to results obtained from neutral loci (Beacham et al. 2001; Miller et al. 2001). New technologies, such as high-throughput sequencing, offer promise in efforts to determine whether American Shad populations in Chesapeake Bay (historical or contemporary) exhibit differences in adaptive variability. New technologies such as high-throughput sequencing and restriction-site-associated DNA markers (RADseq; Baird et al. 2008) offer promise in efforts to determine whether American Shad populations in Chesapeake Bay (historical or contemporary) exhibit differences in adaptive variability. For example, Corander et al. (2013) showed that Baltic Sea populations of Atlantic Herring Clupea harengus exhibiting an $F_{ST}$ of approximately 0.005 with neutral markers showed elevated population differentiation ($F_{ST} = 0.128$) at single-nucleotide polymorphism (SNP) loci identified using RADseq.

Brown et al. (2000) hypothesized that because the James and Pamunkey River populations were genetically divergent before supplementation, replenishment of the James River population with Pamunkey River stock would be detectable in post-supplementation samples as heterozygote deficiencies (Wahlund effects). We found that most samples deviating from HWE were from the James and Pamunkey rivers (both supplemented), and most were post-supplementation samples collected from 2000 onward, shortly after the first Pamunkey River hatchery recruits were detected in the James River in 1997. However, when individual loci were examined within each population, universal deviation among all loci—as would be expected from a Wahlund effect or inbreeding—was not observed. Usually, a range of one to five loci deviated from HWE, and inclusion of some data (locus Asac249) with probable null alleles may have introduced bias into some of our HWE results. Although the precise direction of such bias in our data is unknown, simulation studies provide guidance as to the potential effects. Carlsson (2008) showed that (1) the impact of null alleles is generally small for assignment tests that use HWE expectations for assignment probabilities, even at loci with very high frequencies of null alleles; and (2) existing population differentiation and the number of loci used are more important factors in the accuracy of results.

Past studies employing mitochondrial DNA for mixed-stock analyses of Atlantic coast American Shad populations (Epifanio et al. 1995; Brown et al. 1999) found that allocation of individual fish to their river of origin would be correct 28% of the time. This was a considerable improvement over the random allocation of 6.67% among the 15 source populations (i.e., $K = 15$) that were drawn from a wide geographic area. Within Chesapeake Bay, however, genetic differentiation among the James, Pamunkey, and Rappahannock River populations as measured by AMOVA was essentially zero, except in comparisons with the Susquehanna River population (Epifanio et al. 1995); we obtained the same result in the present study using the more polymorphic microsatellite markers. All of the work to date, including a concurrent microsatellite study by Hasselman et al. (2013), indicates that distinguishing among Chesapeake Bay tributary populations of American Shad will continue to be hindered by low among-population resolution unless more discriminating markers are discovered. Recent studies of salmonid population structure and mixed-stock analyses using either outlier microsatellite loci (Russello et al. 2012) or SNP loci (Bourret et al. 2013) have demonstrated marked improvement over the use of neutral microsatellite loci, and similar methods should be explored for the management of American Shad.

Thus, the question still remains: what is the origin of untagged American Shad recruits in the James River? The most parsimonious hypothesis is that the rapid increase in the number of hatchery returns through 2002 signaled high recruitment of hatchery fish and declining numbers of native James River American Shad. The subsequent, albeit brief, upsurge of untagged returns in 2003–2006 was then likely attributable to returns of the progeny of naturally spawning hatchery fish. Results of hierarchical AMOVA reinforce this hypothesis because post-supplementation James and Pamunkey River samples showed no population differentiation as opposed to the very subtle differentiation between the pre-supplementation James and Pamunkey River populations. Therefore, we cautiously hypothesize that the present James River American Shad population is largely Pamunkey River derived and is a result of the hatchery effort.

Considering that many American Shad populations are in decline despite extensive supplementation efforts (ASMFC 2007; Limburg and Waldman 2009), it has been argued that supplementation is not particularly beneficial for the contemporary American Shad (Hasselman and Limburg 2012). Often in supplemental stocking programs, an inequitable share of funding and attention is given to supplementation, thereby detracting from efforts to address the proximal causes of the declines, such as habitat degradation and a lack of access to spawning sites. For example, in the James River, persistence of the American Shad population appears to be dependent upon hatchery replenishment (Hilton et al. 2011), whereas access to historical spawning habitat remains limited (Aunins et al. 2013). Thus, although supplementation may continue to be an important component for
sustaining the James River American Shad population, supplementation does not appear capable of creating a self-sustaining population in the absence of more rigorous habitat improvements. Periodic genetic monitoring will be a valuable means to continue assessments of supplementation effectiveness in an adaptive management context (Schwartz et al. 2007). Nevertheless, we recommend that the most prudent and effective management approach for American Shad restoration is to focus on preservation of distinct genetic populations where they still exist, combined with efforts to improve habitat quality and to ensure that fishways are effective for providing access to the spawning grounds.

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