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APPROVAL SHEET

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the requirements for the degree of

Doctor of Philosophy

Jennifer Michelle Rhode

Approved 2002

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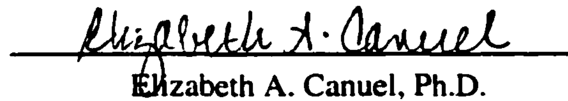

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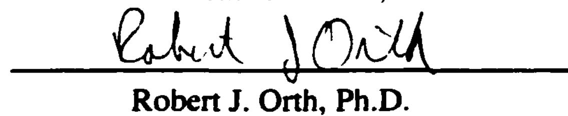

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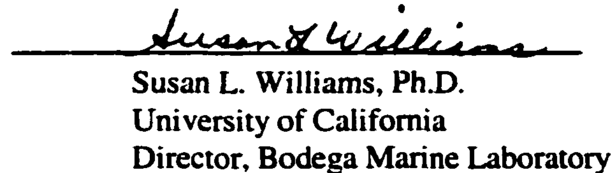

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Microevolutionary Processes in Chesapeake Bay (Virginia, USA) Eelgrass, *Zostera marina* L

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

---

by
Jennifer Michelle Rhode
2002

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DEDICATION

This tome is dedicated first to my family, whose love and support made possible its completion. Richard V. Rhode, Jr., Judith Harrison Rhode, Kristen Juliana Rhode, Emily Elizabeth Rhode, Rosemarie Harrison, Matthew Rhode, Steven Harrison, and many others spent time with me in the field, at the lab bench, and on the telephone. Richard V. Rhode Sr., John Joseph Pershing Harrison, and Elizabeth May Rhode lent support from afar. Marc Harrison ensured that my Ph.D. process seemed short.

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Next, I dedicate this dissertation to the important teachers in my life, especially Rebecca Benton, Joan Withers, Nancy Blizzard, Cecil Short, and Michael Glaser.

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ABSTRACT

Eelgrass (*Zostera marina* L.) is the northern hemisphere's dominant marine angiosperm, a species with both ecological and economic importance. Initial allozyme surveys of eelgrass populations in Chesapeake Bay (Virginia, USA) revealed substantial amounts of geographically-partitioned genetic variation, which could be the result of nonselective demographic processes, including founder events and drift. However, strong spatial variation in the environment and in eelgrass morphology suggests that differential adaptation of isolated beds to local environmental conditions could also produce these patterns. This dissertation used three sets of studies to investigate microevolutionary processes might produce the observed variation among Chesapeake eelgrass beds: 1) an allozyme survey of genetic diversity within and among twelve beds of different ages and sizes, 2) controlled breeding experiments to characterize the mating system of *Z. marina* and determine its susceptibility to inbreeding or outbreeding depression, and 3) reciprocal transplants to test for local adaptation within *Zostera marina* demes. Results showed considerable genetic diversity within beds and strong differentiation among beds but no relationship between genetic diversity and bed age or size, suggesting that founder events or clonal competition do not strongly depress genetic variation in this system. Artificial matings revealed no evidence of inbreeding depression in the 3 beds tested; seed production was significantly higher in selfed crosses than in either outbred or within-bed (inbred) crosses. Finally, reciprocal transplants showed some evidence of local adaptation in shoot density and seed production, but this was inconsistent in space and time. Phenotypic plasticity, perhaps bounded by genetic constraints, appeared to be the

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primary means by which Chesapeake eelgrass responded to local environmental variation. These studies support the emerging idea that eelgrass is not a panmictic obligate outbreeder, and they support important influences of non-selective processes (restricted gene flow and phenotypic plasticity) on the population structure of Chesapeake Bay eelgrass.

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Microevolutionary Processes in Chesapeake Bay (Virginia, USA) Eelgrass, *Zostera marina* L

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Chapter 1: Introduction

Evolutionary Theory and Application

Microevolution

Evolution is a change in allele frequency or chromosome number within a population or species over time. This change may be adaptive, neutral, or maladaptive. Speciation events or evolutionary changes over geologic time scales are termed macroevolution. Shifts in gene frequencies within a few generations constitute microevolution. Microevolution is the result of mutation and migration, which increase genetic variation, and selection, drift, and founder events, which reduce genetic variation. The relative influence of each evolutionary force may be discerned using long-term genetic monitoring, genetic and environmental correlations, and *in situ* manipulations (Endler 1986). A review of plant population studies (Linhart and Grant 1996) concluded that most genetic substructure in these demes is the result of natural selection rather than non-selective processes. Microevolutionary forces, acting in concert, in opposition, or alone, dramatically impact the distribution of genetic diversity within and among populations. Elucidation and analysis of microevolutionary processes can provide insight into the intimate coupling between organisms and their environments.

The rate of microevolutionary change depends on the forces driving it and the genetic variation on which it can act. Microevolution is accelerated in populations with sexual reproduction, short generation times, and small population sizes. Geographic fragmentation of a population also increases its rate of microevolution.

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Microevolutionary changes may occur most quickly in populations that experience dramatic, unpredictable environmental fluctuations. For example, plant populations can shift allele frequencies within a decade following anthropogenic environmental changes such as eutrophication (Snaydon and Davies 1982) or heavy metal contamination (M^c^Neilly 1967). Genetic changes occur more slowly in asexual populations or those whose individuals have lots of phenotypic plasticity (Cheplick 1991).

Genetic diversity and structure

Evolution results when natural selection acts on a population's pre-existing genetic diversity; the amount and nature of a population's genetic variation can therefore predict its ability to adapt (Fisher 1958, Beardmore 1983). Studies of genetic diversity and structure have historically relied on data from allozymes, proteins produced as allelic alternatives of a single gene (Murphy *et al.* 1996). Electrophoresis is used to separate these proteins by weight and charge, usually in a gel matrix, and differences in final band position are scored as polymorphisms. Allozymes are useful in studies of large-scale population structure where elucidating fine-scale genetic patterns is not necessary.

Local adaptation and phenotypic plasticity

Local adaptation occurs when differential selective forces produce divergences among populations. It is often found across environmental gradients or between habitat types and results in different genotypes having higher fitness in different parts of the species' niche. Over time, local adaptation may promote speciation. The degree of local

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adaptation depends on the extent of gene flow, intensity of selection, mode of inheritance, and amount of genetic variation within the meta-population (Linhart and Grant 1996). Disturbances of locally adapted populations or their niches can result in extirpations, particularly if the relationship between a species' genotype and habitat is unique.

Populations that are highly genetically structured may be locally adapted. Plant populations, whose sedentary adult stages marry them to fluctuating or evolving environments, are often locally adapted (Bradshaw 1984, Schmitt and Gamble 1990). The process of local adaptation may occur over periods of centuries or less than a decade (Wu *et al.* 1975, Snaydon and Davies 1982). Local adaptation can occur even in the presence of gene flow and has been observed between plant communities separated by distances from many kilometers to a few centimeters (Linhart and Grant 1996). The most common way to test local adaptation is with a series of reciprocal transplants, which move organisms from native to foreign habitats and pair different phenotypes in common sites (Schemske 1984, Schmitt and Gamble 1990). The survival and performance of natives and foreigners are monitored and compared, with enhanced performance of an individual in its home territory considered evidence of local adaptation.

Many plant populations respond to environmental changes with phenotypic plasticity rather than local adaptation (Sultan 2000). Plasticity might incur a genetic cost, though, and plastic plants might be outcompeted in stable environments. Plasticity is also more difficult to retain than local adaptation, because it requires selection across multiple genotypes by a host of environmental conditions.

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Reproductive strategies and demographic constraints

The mating system of sexually reproducing organisms ranges from outcrossed to highly inbred; these systems are maintained by an array of physiological, behavioral, and geographic mechanisms. Outbred matings occur between unrelated individuals or between individuals from different populations (Waser 1993a). Outbreeding homogenizes genetic structure; its advantages include heterosis and masking potentially deleterious recessive alleles. However, breaking multilocus allelic associations and disrupting local adaptation can result in reduced offspring fitness, or outbreeding depression. This phenomenon has been experimentally demonstrated with artificial matings of several terrestrial plant species but seems to be less common than inbreeding depression (reviewed in Waller 1993 and Waser 1993b).

Inbred matings occur between kin who share alleles identical by descent, such as related individuals from a single population (Waser 1993a). Populations which are sessile, are physically fragmented, or have limited gamete and offspring dispersal distances often inbreed (Waser 1993b). Without genetic exchange, inbreeding populations can become genetically distinct from adjacent populations, with either positive or negative consequences. Consanguineous matings may increase parents' genetic representation in the next generation, preserve coadapted gene complexes, maintain local adaptation, and provide a mechanism for mutational purging (Waller 1993). Conversely, inbreeding can also result in reduced heterozygosity and increased expression of deleterious recessive alleles within a population. The decrease in offspring

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fitness, or inbreeding depression, is common among animals and has been detected in several terrestrial plant species (reviewed in Waser 1993b).

Self-fertilization is an extreme form of inbreeding in which the genome of a hermaphrodite parent recombines with itself. This strategy maximizes parents' contribution to their offspring and may be selected for in stable environments; it provides reproductive assurance while retaining a system for outbreeding (Waser 1993a). Selfing also avoids recombination with non-adapted genomes. In heterozygous individuals, self-fertilization can generate limited variability. However, organisms that self-fertilize can experience many disadvantages of inbreeding, including the accumulation of deleterious mutations and reduced heterozygosity. Life history characteristics, including the chronology of gamete maturity, affect the frequency of self-fertilization in hermaphrodites. Asynchronous flowering may prevent selfing and promote outcrossing (Charlesworth and Charlesworth 1978), while simultaneous flowering allows the reverse.

Demography may constrain populations from engaging in their most adaptive mating system (Shields 1993, Waser 1993a). Changes in the physicochemical or biotic environment that alter dispersal patterns affect mating systems and thus a population's fitness. Population fragmentation can prevent long-distance exchange of gametes or individuals and force the population to inbreed. This fragmentation may be physical, such as that caused by extinction or founder events, or temporal, such as microhabitat-induced differences in flowering time (e.g. Stanton and Galen 1997). Alternatively, the removal of physical barriers to gene flow may allow previously separate populations to

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exchange gametes. This often results in outbreeding depression, but it can be advantageous, providing genetic variation that is beneficial to long-term survival.

Study Organism

Seagrasses, of which there are fifty-eight described species in twelve genera (Larkum *et al.* 1989), are of tremendous ecological and economic importance. They consolidate and stabilize sediments and provide food and shelter for diverse fauna. Seagrasses consolidate and stabilize sediments, provide food and shelter for diverse fauna, recycle nutrients, improve water quality, and serve as nurseries for many commercially important species (M[c]Roy and Helfferich 1977, Larkum *et al.* 1989). Costanza *et al.* (1997) estimate that seagrass beds provide $19,004/hectare/year in ecosystem services.

Aquatic angiosperms possess a combination of characteristics that make them interesting subjects for population genetic and mating system studies. Individual plants can include both clonal (rhizomatous and vegetative growth) and sexual (selfing and outcrossing) components in their life histories; environmental and genetic factors determine the actual mode of propagation. Water is a unique and directional dispersal vector, and it can influence the degree of genetic structure (Williams and Orth 1998) found in seagrass populations. Gene flow in seagrasses is probably also limited by the patchy structure of aquatic habitats (Barrett *et al.* 1993).

This dissertation investigates questions of genetic structure and local adaptation in *Zostera marina* (eelgrass), the most common temperate seagrass. Extensive beds are

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ubiquitous in intertidal and sub-littoral soft-bottom communities throughout the northern hemisphere (McRoy and Helfferich 1977). An eelgrass individual consists of one or several genetically identical, physiologically integrated leaf bundles connected by a subterranean rhizome. Shoots and their associated rhizome are termed a ramet, and shoots which are genetically identical are termed a genet, regardless of physiological connections. *Z. marina* shoots grow from a basal meristem, with the inner, younger blades in a ramet growing faster than the outer, senescent blades. Shoot density is controlled by many factors, including current regime, biotic activity (Zimmerman *et al.* 1996), epiphytes (Hauxwell *et al.* 2001), and light levels (Backman and Barilotti 1976) (Jernakoff *et al.* 1996, Hemminga and Duarte 2001).

Eelgrass disperses by movement of pollen (de Cock 1980), seeds (Orth *et al.* 1994), reproductive shoots (den Hartog 1970, Harwell 2000) and, rarely, vegetative fragments (Ewanchuk and Williams 1996). Measurements of pollen and seed dispersal in a tidally-dynamic Washington, USA population used artificial deployments to show that these gene flow vectors traveled only 1.1 to 1.27 m from their source (Ruckelshaus 1996). Other investigators have concluded that eelgrass seeds are stationary once released from maternal tissue (Orth *et al.* 1994). If these measurements are broadly applicable to *Zostera marina* populations, demographic factors may severely constrain gene flow, particularly in fragmented demes. Eelgrass populations might not be able to outbreed (exchange pollen among beds) due to demography, and thus inbreeding might be more common than eelgrass' protogynous reproductive mode would suggest. Alternatively, Harwell (2000) suggested that reproductive shoots might travel up to 30

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km from their source, and his data showed new beds established up to 100 km from the nearest potential source bed. This implies that gene flow is relatively broad.

Focal Populations

Populations of *Zostera marina* in Chesapeake Bay (Virginia, USA) were used to address general questions about genetic diversity, local adaptation, and mating systems. *Z. marina* occupies a large geographic and ecological range in Chesapeake Bay, exhibiting a wide array of morphologies over small spatial and temporal scales (Orth and Moore 1986). Eelgrass survives a variety of salinity, temperature, light, and disturbance regimes, and populations are often exposed to drastic environmental fluctuations in a genet's lifetime. Salinity at a single location can vary as much as 8 psu annually, while water temperatures range from 0 to greater than 30°C (Wetzel and Penhale 1983). In response to this variability, selection for eurytolerance may occur within Chesapeake Bay eelgrass populations.

The well-documented annual cycle of Chesapeake *Zostera marina* growth and reproduction (Orth and Moore 1986) is composed of several distinct phases. In a typical year, seeds germinate in October or November, and standing stocks of eelgrass remain low until March. Shoot density begins to increase in March and peaks in June and July. Between April and June, reproductive shoots are found in abundance (Orth and Moore 1986), and the seeds that they bear mature in late spring. From July until September, the combination of temperatures >25 °C and low light from increased phytoplankton blooms causes mature plants to defoliate (Moore *in* Batiuk *et al.* 1992). Newer blades,

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root/rhizome, and seeds from the previous spring survive to revegetate beds beginning in mid-September. *Z. marina*'s vegetative growth cycle is biphasic, with growth maxima in midsummer and late fall (Moore *in* Batiuk *et al.* 1994).

Chesapeake Bay populations of *Zostera marina* experienced two demographic bottlenecks in this past century. The 1930's outbreak of *Labyrinthula* sp., a pathogenic slime mold, was implicated as the causal agent in the death of eelgrass populations bay-wide (Rasmussen 1977). Most areas recovered quickly from this wasting disease, but some ocean lagoons along the Delmarva Peninsula never became revegetated (Short *et al.* 1987). Increased anthropogenic pressures in the wake of Tropical Storm Agnes (1972) further decimated many Chesapeake Bay eelgrass populations (Orth and Moore 1983); some have yet to recover fully (Orth *et al.* 1994). Reestablishment of these populations may have been slowed by demographic constraints, including dispersal limitation, but lack of suitable habitat might also constrain bed recovery.

Microevolution in Eelgrass Populations

Allozyme studies of eelgrass populations from California (USA), Rhode Island (USA), Mexico, and the Netherlands revealed little genetic diversity (Gagnon *et al.* 1980, M[c]Millan 1982, Heij and Nienhuis 1992). This, combined with observations of rapid vegetative growth and low rates of flowering in some populations (Phillips *et al.* 1983), led biologists to deduce that this species was primarily clonal (M[c]Millan 1982) and adapted by means of phenotypic plasticity. In fact, the lack of observed genetic diversity may have been an artifact of inadequate genetic analyses. More recent studies have used

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additional allozyme loci or molecular techniques and found that eelgrass beds display substantial genetic and phenotypic substructuring both within and between patches (Laushman 1993, Alberte *et al.* 1994, Ruckelshaus 1996, Williams and Orth 1998). The nature of this variation (adaptive or not) has been established for few populations, though.

Population structure in eelgrass beds could be attributable to genetic drift. Cox *et al.*'s (1992) study of eelgrass fertilization demonstrated that pollen was viable for up to 7.7 hours after release from its source plant, that eelgrass flowering times were staggered across a tidal gradient, and that genetic differences between intertidal and subtidal beds were highly significant. In the face of restricted migration, demographic isolation might allow genetic interpatch differences to become established. The authors surmised that short-lived pollen and asynchronous flowering combined to isolate eelgrass beds; drift and founder effects then contributed to the observed genetic structure. The possible role of selection was not rigorously tested, however.

The population structure of *Zostera marina* may also be influenced by founder effects. The founder effect is a reduction in genetic diversity observed in small, new populations, whose colonizing members represent only a fraction of their parent population's gene pool. This dearth of genetic diversity may be amplified by the clonal component of eelgrass life history. Although *Zostera marina* takes two years to attain sexual maturity, new patches expand rapidly in their first year. This, combined with the low chance of seedling establishment (Churchill 1983, Hootsmans *et al.* 1987), makes it probable that clonal growth of founding individuals has a strong effect on a population's

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genetic composition. The presence of founder effects in eelgrass beds has yet to be examined thoroughly.

Finally, selection and/or non-selective disturbance in the form of physical or mechanical stress, inter- and intraspecific competition, pathogens, or environmental heterogeneity may create patch structure through differential survival of individuals (e.g. Roy 1993). Selective forces that vary in space can promote the development of local genetic adaptation. Allozyme and reciprocal transplant data suggest that there is local adaptation among west coast populations of *Zostera marina* (Ruckelshaus 1994; 1996). The degree of local adaptation among Chesapeake populations of eelgrass is unknown but has potentially important implications for management and restoration efforts. If beds are locally adapted, restoring extirpated beds might be difficult or impossible, and supplementing existing beds with foreign plants, while increasing total coverage, might cause beds irreparable, long-term genetic harm.

Genetic Diversity in Seagrasses

The potential importance of genetic diversity to the persistence of seagrass populations has been alluded to (Alberte *et al.* 1994, Reusch 2001), and there are some data that support this hypothesis. Ruckelshaus (1995) demonstrated local adaptation of Washington, USA *Z. marina* to inter- or sub-tidal sites. Evidence collected thus far indicates that genetic diversity may not be crucial to the survival of other seagrass species. An allozyme (14 loci) and RFLP study by Waycott *et al.* (1996) found no genetic diversity in thirteen highly-productive, persistent populations of *Amphibolus*

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antarctica sampled over a wide geographic range. Of course, the success could be due to the phenotypically plastic response of these genetically uniform populations to novel environments, and the effects of future stresses on genetically uniform populations cannot be predicted *a priori*. The role of genetic diversity in seagrass fitness is thought to be important (Williams 2001) but is not well-understood and cannot be generalized across taxa. Long-term genetic monitoring of many seagrass species and populations using large sample sizes might further clarify the role of genetic diversity in these plants (Alberte *et al.* 1996).

Genetic diversity and structure in eelgrass populations

Numerous studies have used allozymes and molecular genetic markers to examine the genetic diversity and structure of Zostera marina populations. RFLP analysis of the 17S and 28S rDNA genes was used in one such study (Fain *et al.* 1992). This technique revealed little genetic diversity, leading the authors to conclude that physically separated eelgrass populations were genetically similar. It is important to note that the authors used samples from populations which might have naturally low genetic diversity (McMillan 1982) and that the researchers analyzed relatively conserved portions of rDNA. Alberte *et al.* (1994) found genetic differences both within and between three California (USA) eelgrass populations using multilocus RFLPs.

Other investigators have examined relationships between genetic and demographic variables in eelgrass populations. Williams and Davis (1996) did a correlational analysis of the size, age, and genetic (allozyme) diversity of eelgrass beds in

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California (USA) and Mexico. The investigators designed their experiment conservatively, collecting the same number of samples per location. They found no relationship between age and genetic diversity of these populations but did find that larger beds had significantly more genetic diversity than beds with less areal coverage.

Ruckelshaus (1996) used a variety of approaches to examine the role of evolutionary forces in creating population structure within eelgrass beds in Washington, USA. She used traps to determine that the average pollen dispersal distance was 1.1 m in these high-energy environments; she used seed and seedling censuses to estimate a mean seed dispersal distance of 1.27 m. Ruckelshaus then elucidated subpopulations' mating systems by performing electrophoretic analyses on seed embryos. From these demographic and genetic data she calculated the genetic neighborhood area, N_a, to be 521 m^2 and the average neighborhood number, N_b, to be 6812. Ruckelshaus concluded that the neighborhood size of the experimental population was large enough to preclude genetic drift (Wright 1946) and speculated that the observed genetic structure was due to local selection and adaptation.

Reusch *et al.* (1999a) used microsatellites to examine genetic structure in European eelgrass populations. They found that most clusters of clones were products of vegetative spread rather than inbreeding. In a later study, Reusch *et al.* (1999b) found that populations once thought to be genetically homogenous actually had lots of genetic diversity both within and among populations. Reusch *et al.* (2000) used microsatellite loci to show that most Baltic populations of *Zostera marina* were in Hardy-Weinberg equilibrium. From this, he concluded that eelgrass in these populations reproduced by

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outcrossing and that pollen, seeds, and shoots were exchanged freely among populations. He also found significant correlations between genetic and geographic distances.

In 1998 Williams and Orth used allozyme electrophoresis to survey inter- and intrapatch diversity of nine transplanted and natural *Z. marina* populations in Chesapeake Bay (Virginia, USA). The authors found an average F_{ST} value of 0.335 for natural eelgrass populations, indicating that the diversity of this species is strongly partitioned among many subpopulations. They also found that more than 70% of genetic differences were among rather than within *Zostera marina* beds. The causes and consequences of this genetic population structure have yet to be characterized. Finally, the authors found that transplanted beds retained the genetic signature of their donor beds. Genetic distances (Nei 1972) between transplants and their source populations were very short. As in other transplanted eelgrass beds (Williams 2001), up to fifteen years of post-transplantation seed recruitment seem to contribute little to the genetic diversity of these beds; genetic diversity of transplanted beds was reduced compared to donor beds.

Local adaptation in eelgrass populations

Locally adapted populations exhibit reduced fitness when removed from their native habitat. Transplant data and field observations indicated that seagrasses might undergo phenotypic changes and maintain constant fitness (Setchell 1927, Gagnon *et al.* 1980). *Zostera marina*'s high degree of phenotypic plasticity was also thought to preclude local adaptation. Eelgrass can accommodate a wide range of environments through phenotypic plasticity, though genetics also influence its performance and

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constraint the degree of phenotypic plasticity. Eelgrass restoration projects typically assume that *Z. marina* populations are not locally adapted (Williams and Orth 1998).

Previous investigators have used reciprocal transplants of *Zostera marina* shoots to elucidate genotype/environment relationships (Phillips and Lewis 1983, Dennison and Alberte 1986, Backman 1991, Phillips 1996). McMillan and Phillips (1979) reciprocally transplanted eelgrass shoots between Puget Sound, Washington and Izembek Lagoon, Alaska. One year after planting, native plants in both Puget Sound and Izembek Lagoon flowered. Transplants from Puget Sound to Izembek Lagoon also flowered, but shoots from Alaska did not flower in Washington. Morphological data also indicated that blade width changed with planting environment; while width of transplants approached the width of natives, these values never completely converged. Though there seemed to be some evidence of local adaptation, the use of adult shoots in transplantation means that the observed retention of morphological characteristics might have resulted from canalization. van Katwijk *et al.* (1998) transplanted *Z. marina* from five European populations into common garden tanks. They found that populations maintained their reproductive strategy (semi-annual or perennial) after transplanting. They found a source effect on below-ground biomass, which they assumed was genetic, but there was no source effect on above-ground biomass.

Other investigations transplanted shoots rather than seeds, eliminating the possibility of trait canalization. Ruckelshaus (1994) used reciprocal transplants of both seeds and adult shoots to test for local adaptation along a tidal gradient in a single Washington (USA) population. Seeds showed greater germination, survival, and growth

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rates in their native habitats, indicating that they were locally adapted. Adults did not show any difference in survival between native and foreign environments. Although there was local adaptation, selection acted only on early life history stages, so adults from different source populations had equivalent performance. van Lent and Verschuure (1995) examined the relationship between intraspecific variability and environmental factors in four *Z. marina* populations from the southwestern Netherlands. The authors germinated and raised seeds from different populations in common garden tanks. The researchers found differences in morphology and flowering density between individuals from disjunct source populations, suggesting variation in fitness. This variation may have been attributable to underlying genetic diversity, although they had no genetic data to confirm this.

Eelgrass mating systems and isolation by distance

The mechanisms of *Z. marina* propagation have been studied for decades (Setchell 1929, den Hartog 1970, de Cock 1980, Cox *et al.* 1992), but the relative importance of vegetative and sexual reproduction has yet to be established. Eelgrass seeds (Fishman and Orth 1996) and seedlings (Hootsmans *et al.* 1987) can have high rates of mortality, while rates of lateral expansion and shoot production for established genets in Denmark average 16 cm/y (Olesen and Sand-Jensen 1994b) and 0.97 shoots/y (Olesen and Sand-Jensen 1994a). This, combined with an apparent dearth of genetic diversity (Gagnon *et al.* 1980, McMillan 1982, Heij and Nienhuis 1992) and abundance of clonal reproduction (Reusch *et al.* 1999a), supported the assumption that *Z. marina*

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propagation was mainly vegetative (Les 1988). Studies of reproductive physiology and dynamics (Cox *et al.* 1992) demonstrated that *Zostera marina* could and did reproduce sexually but concluded that most of these matings were inbred.

Observations of flowering asynchrony in *Z. marina* led researchers to conclude that eelgrass outcrossed (Setchell 1929, de Cock 1980). Recent evidence shows that this is not always the case (Cox *et al.* 1992, Ruckelshaus 1995) but supports the importance of outbreeding in *Z. marina* populations. Ruckelshaus (1995) used allozyme analyses of seeds to estimate the mating system of a single Washington, USA *Z. marina* population over two breeding seasons. This population was genetically substructured along a tidal gradient. Her data showed that these plants were mainly outbreeding, with rates of outcrossing (t), or genetic exchange between inter-and subtidal plants, ranging from 0.611 to 1.000. Ruckelshaus also used a series of artificial breedings to determine if these plants experienced inbreeding depression, calculated as relative performance (RP). She found that inbred matings produced lower seed set (RP = 0.205 to 0.300) and survival (RP = 0.131 after 7 months) than outcrossings. She concluded that, in spite of some flowering synchrony, selection maintained outcrossing in this population.

Because eelgrass occupies a broad range of habitats over a vast geographic expanse, the applicability of Ruckelshaus' study to other systems is unknown. The mating systems of some *Z. marina* demes may be influenced by demographic constraints such as limited pollen and seed dispersal. Historically fragmented eelgrass populations, including those in Chesapeake Bay (Virginia, USA) (Orth and Moore 1983), may have mating systems which are dictated by demographic (e.g., plant proximity, pollen

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dispersal distances) rather than fitness considerations. Inbreeding depression may not be universal in this species; in fact, genetically homogenous or locally adapted eelgrass populations may be capable of inbreeding without adverse fitness consequences. It is also possible that genetically structured *Z. marina* populations such as those in Chesapeake Bay (Williams and Orth 1998) experience outbreeding depression.

Objectives and Hypotheses

This dissertation explores microevolutionary processes that create eelgrass population structure in Chesapeake Bay and its tributaries. These questions have broad genetic, ecological, and evolutionary applications across local, regional, and global scales. This work had three main goals. First, genetic diversity and structure within Chesapeake eelgrass beds was surveyed, and relationships between genetic and demographic variables were explored. Next, the breeding structure of Chesapeake Bay eelgrass was explored to determine whether or not these populations outbreed. Finally, populations of Chesapeake Bay eelgrass were tested to see if they exhibited local adaptation.

Twelve Chesapeake Bay eelgrass beds were surveyed to determine the relationship between bed age, bed size, and genetic diversity and structure. This survey included beds of 1) similar sizes but different ages and 2) similar ages but different sizes. Genetic diversity within and among beds was estimated using allozyme analyses. Correlative statistics were used to test for relationships among genetic diversity and bed age/size. Null hypotheses were as follows:

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H_{o1}: Levels of genetic diversity within old (> 65 years) and young (< 7 years) beds are comparable.

H_{o2}: Levels of genetic diversity within large (>100 ha) and small (< 10 ha) beds are comparable.

H_{o3}: Patterns of genetic diversity within old (> 65 years) and young (< 7 years) beds are comparable

H_{o4}: Patterns of genetic diversity within large (>100 ha) and small (< 10 ha) beds are comparable.

To determine whether Chesapeake Bay eelgrass experiences inbreeding or outbreeding depression, a set of artificial breedings was conducted using plants from three sites.

H_{o1}: Seed set from selfed, inbred and outbred matings is comparable.

Environmental differences among sites may contribute to the development of local adaptation in *Zostera marina* populations. To test for local adaptation in these demes, shoots and seeds from geographically, ecologically (preliminary data; this study), and genetically (Williams and Orth 1998) different regions were transplanted reciprocally. Shoot and seed survivorship and performance were monitored over two years. Phenotypic convergence of native and transplanted shoots was also measured.

H_{o1}: Chesapeake Bay eelgrass shows no evidence of local adaptation.

Some of these questions have been addressed for other species or systems, but the unique characteristics of *Zostera marina* and Chesapeake Bay make them worthy of

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investigation. Eelgrass is one of the few monoecious seagrass species, and it is the most widely-distributed of all submersed angiosperms (Larkum *et al.* 1989). Chesapeake Bay is near the southern limit of *Zostera marina* distribution. Its waters are subject to extreme fluctuations in temperature, light, and nutrients, and eelgrass populations in the Chesapeake have been the subject of extensive monitoring since the 1930s. The studies presented here will prove particularly important in the creation of management strategies for qualitative and quantitative seagrass preservation (M^c^Roy 1996).

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Chapter 2: Relationships between Population Age, Size, and Genetic Structure in Chesapeake Bay (Virginia, USA) Eelgrass (*Zostera marina* L)

ABSTRACT

A population's genetic structure and diversity can reveal the demographic and selective forces to which it has been exposed and influence that population's response to environmental changes. Genetic structure can also help explain differences among populations in individual morphology or fitness. Beds of eelgrass (*Zostera marina* L) in Chesapeake Bay (Virginia, USA) are marked by significant variation in shoot density and morphology. They have also experienced substantial reductions in recent decades, and these might have important implications for genetic structure and connectivity among beds. Chesapeake eelgrass beds are marked by significant variation in shoot density and morphology. Following a previous allozyme survey that revealed substantial geographically-partitioned genetic variation, this study examined morphological and genetic (allozyme) structure and diversity within and among eelgrass beds of different ages (> 65 years and < 6 years) and sizes (> 100 ha and <10 ha) to investigate the influence of known population history on genetic structure of this clonal plant. While there was strong and significant morphological variation among individual beds, no morphological measure varied consistently among the three bed types (old and large, old and small, young and small). Similarly, despite strong genetic differentiation among beds (F_{ST} = 0.198), much genetic diversity was found within beds, and the amount of diversity differed little by bed age or size. Beds showed significant levels of inbreeding

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(mean $F_{IS} = 0.68$ over all beds), but inbreeding in old, small beds was significantly lower than in other bed types. There was no clear sign of isolation by distance at the scale of this study, as there was no relationship between genetic and geographic distance, nor was genetic distance related to morphological distance. These results suggest that local environmental conditions have a greater influence on plant morphology than do bed age or size, and they support the hypothesis that new eelgrass beds are established by multiple founder genotypes and are maintained with little loss of genetic diversity over time periods greater than 65 years.

keywords: *Zostera marina*, allozyme, genetic diversity, morphology, age, size, Nei's genetic distance, F_{ST}

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INTRODUCTION

Levels and distribution of genetic diversity influence populations profoundly. Genetically diverse populations are better able to adapt to environmental changes, while those with lower diversity are more vulnerable to extinction because they are less likely to contain genotypes adapted to the new conditions (Beardmore 1983). The diversity and distribution of genotypes can provide information about a population's history, including disturbances, demography, local adaptation, selective events (Van Dijk 1987, McCauley *et al.* 1995, Harada and Iwasa 1996, Linhart and Grant 1996), and the relative success of sexual and vegetative reproduction (Harada *et al.* 1997), an especially important measure in clonal plants. Disturbance and selection tend to decrease intrademe diversity, while immigration and other forms of gene flow can enhance a population's genetic diversity. Genetic structure can be reflected in differential morphology, performance, or fitness, or it might be apparent only in the distribution of neutral molecular markers (Endler 1986).

Patchy habitat distribution, in combination with selective forces such as physical or mechanical stress, inter- and intraspecific competition, pathogens, or environmental heterogeneity, often creates population structure through differential survival of individuals (Laska 2001). Selective forces that vary in space can promote the development of local genetic adaptation, even over small distances (McNeilly 1967, Joshi *et al.* 2001), although populations may respond to changing conditions via phenotypic plasticity rather than genetic changes (Cheplick 1991). A population's genetic diversity can also be affected by demographic variables, including its age or size (Wright 1978, Oostermeijer *et al.* 1994, Weidema *et al.* 1996). Older populations might be expected to

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harbor more genetic diversity because may contain more individuals, or they have had more time to receive immigrants. Alternatively, their diversity might be reduced over long periods by selection (Beardmore 1983). Because population age and size are often correlated, it can be difficult to differentiate between age and size effects, and these effects can be obscured in clonal plant populations (Eriksson 1993).

Eelgrass (*Zostera marina*) is an interesting subject for studies of population genetic structure because of its great economic value (Costanza *et al.* 1997), broad distribution (den Hartog 1970, McRoy and Helfferich 1977), multiple reproductive strategies (Orth *et al.* 1994; Ruckelshaus 1994; Ewanchuk and Williams 1996; Harwell 2000; Rhode 2002, Chapter 3), dispersal via both shoots and seeds (Harwell 2000), and morphological diversity. The influence of genotypic and environmental factors on eelgrass phenotype, performance, and fitness is of particular interest. Initial allozyme studies of eelgrass population genetics in North America and Europe concluded that eelgrass populations contained very little genetic diversity (Gagnon *et al.* 1980, McMillan 1982, Heij and Nienhuis 1992). Observations of rapid vegetative growth, low flowering rates (Phillips *et al.* 1983), and limited dispersal (Ruckelshaus 1996) supported conclusions from the initial genetic data. Researchers concluded that most *Zostera marina* reproduction was clonal (McMillan 1982) and that eelgrass used phenotypic plasticity to adapt to environmental variation. Later studies, which used additional allozyme loci or DNA-based molecular markers (RFLPs, microsatellites) found more genetic and phenotypic substructuring both within and among patches of eelgrass (Fain *et al.* 1992, Laushman 1993, Erikkson 1993, Alberte *et al.* 1994, Ruckelshaus 1996,

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Williams and Orth 1998, Reusch *et al.* 1999b). Such studies also supported earlier hypotheses that clones tend to be large (Reusch *et al.* 1999a) and thus that clonal propagation is important to eelgrass demography.

In Chesapeake Bay (Virginia, USA), several historical events have had strong impacts on eelgrass populations, and these are apparent in their population genetic structure. First, populations of eelgrass went through a probable demographic bottleneck in the 1930's, when an outbreak of *Labyrinthula* sp., a pathogenic slime mold, apparently caused the demise of eelgrass throughout Chesapeake Bay (Rasmussen 1977, Short *et al.* 1987). Later, in the 1960's and 1970's, Tropical Storm Agnes, combined with anthtropogenic eutrophication and high sediment input, further decimated many Chesapeake Bay eelgrass populations (Orth and Moore 1983); some of these have yet to recover fully (Orth *et al.* 1994). Because the size and persistence of Chesapeake Bay eelgrass beds has been monitored for several years, these events, and subsequent ones, provide an opportunity to examine the influence of known population parameters (age, size) on genetic structure in this metapopulation.

In the first genetic survey of Chesapeake Bay eelgrass, Williams and Orth (1998) surveyed nine natural and transplanted eelgrass beds with twelve allozyme loci and found substantial interpopulation variation. F_{st} averaged 0.335 among natural eelgrass beds, indicating strong partitioning of genetic diversity among beds. Several processes could contribute to the observed genetic diversity and structure in Chesapeake Bay eelgrass beds, including founder effects, bottlenecks, and/or clonal competition (selection) under different environmental conditions. Founder effects, the reductions in genetic diversity

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observed in small, new populations, might also be amplified by clonal propagation. The influence of clonal growth on vegetative structure might be particularly strong in a bed's first year, before the founding plants reach sexual maturity (Churchill 1983, Hootsmans *et al.* 1987, Harwell 2000).

Among West Coast populations of *Zostera marina*, allozyme and reciprocal transplant data suggested that eelgrass genotypes were locally adapted along a depth gradient (Ruckelshaus 1994, 1996). In a companion study to that presented here, we found no consistent evidence of local adaptation in Chesapeake Bay eelgrass (Rhode 2002; Chapter 4). Moreover, although strong genetic structure has been observed in Chesapeake Bay eelgrass, observations of seed-bearing shoots suggest that dispersal potential of Chesapeake Bay eelgrass is fairly high (Harwell 2000). Existing beds might be supplemented by regular inputs from foreign seeds and shoots (Harwell 2000) although there is no genetic evidence to support this phenomenon and the frequency of successful seed establishment is uncertain (Moore *et al.* 1993).

This study used a metapopulation of eelgrass (*Zostera marina*) beds of known history to test the influence of bed size and age on patterns of genetic diversity. Because they contained more individuals and had more time over which to receive immigrants, old, large beds were expected to have more genetic diversity and genetic substructure than young, small beds. This study was motivated in part by observations of significant interpopulation differences in eelgrass morphology within Chesapeake Bay. The survey included beds of 1) similar sizes but different ages, and 2) similar ages but different sizes. Allozyme electrophoresis was used to estimate genetic diversity and spatial structure

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within and among beds. First, relationships among genetic diversity, bed age, and bed size were examined. Next, relationships between genetic and morphological differentiation among eelgrass beds were explored. Finally, these genetic data were used to make inferences about demographic forces that structure these populations.

METHODS

Field Sampling

This genetic survey included twelve disjunct *Zostera marina* beds (Figure 1). Aerial photographs and ground monitoring records (US Environmental Protection Agency Chesapeake Bay Program; Orth *et al.* 1998 and earlier reports; R. J. Orth pers. comm.) were used to identify historically persistent beds, designated old (greater than 65 years old), and recently founded eelgrass beds, designated young (less than 7 years old at time of survey). Four recently founded and four historically persistent patches of less than 10 ha areal coverage (small) were included in this survey (Table 1). Though smaller beds are present throughout Chesapeake Bay, they were not used in this study because: 1) small beds might be transient and unlikely to contribute significantly to bed structure, and 2) very small beds are difficult to select randomly because beds less than 1 m in diameter do not appear in aerial photographs. Four old, large (greater than 100 ha areal coverage) patches were also surveyed (Table 1). Areal coverage was assumed to be proportional to the number of individuals within a population. Thus, the total number of beds surveyed included four old, large beds; four old, small beds; and four young, small beds (Figure 1).

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Using GIS (Geographic Information System) technology and aerial photographs from the Virginia Institute of Marine Science's Submerged Aquatic Vegetation mapping laboratory, 100 random, non-clustered GPS (Global Positioning System) sampling points were generated for each eelgrass bed. To maintain a balanced statistical design, the same number of sampling points was used for each bed, regardless of bed size (*as in* Williams and Davis 1996). Sampling points were at least 2 m apart (*as in* Ruckelshaus 1994) to minimize the probability of sampling a single clone more than once (but see Reusch *et al.* 1999a).

In the field, each point was located using a combination of GPS tracking and ground-based triangulation. Eelgrass shoot density was measured by counting individual shoots within a 10 x 10 cm quadrat and extrapolating this to shoots per m^2. At each point a single *Zostera marina* shoot was collected for genetic analysis. This shoot was stored in cool water to preserve protein integrity until laboratory extractions.

All samples were collected within a 5-week period in spring 1998. The restricted time frame was chosen to minimize the chance of observing temporal effects on population genetic structure. Spring sampling was also advantageous because collections were done at the point of maximal population stability, before the generation of newly-formed seeds recruited and before eelgrass's predictable summer defoliation.

Morphometric and Genetic Analyses

In the laboratory, each shoot's number of blades, blade length, and blade width was recorded to the nearest millimeter. The methods of Williams and Orth (1998) were used to extract proteins from each shoot's primary blade. Briefly, blades were rubbed

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with Kimwipes to remove epiphytes and then rinsed in distilled water. A mixture of eelgrass and Cherimoya buffer was ground with a mortar and pestle, and the extract was divided into four aliquots, which were distributed among cell well plates. Quadruplicate protein extracts were stored at –80 °C until electrophoresis. Sample division allowed replicates to be run at multiple times or on different buffer systems without subjecting an individual sample to destructive freeze-thaw cycles.

Subsets of the samples were screened with thirty-four allozyme buffer/stain systems (Soltis *et al.* 1983, Richardson *et al.* 1986, Murphy *et al.* 1996, Williams and Davis 1996, Williams and Orth 1998) to identify systems that produced consistently-scorable bands for these samples (Table 2). Of the 34 systems, seven yielded visible and reliably-scorable bands for all test samples, so these systems were used to test extracts from all 1200 shoots (Table 3). Gels for all stain systems were run under amperage and time conditions identical to those reported in Williams and Orth (1998). After gels had run, they were sliced and stained them according to the methods of Williams and Orth (1998) and Murphy *et al.* (1996). All gel slices were scored and photographed; an autoimage analyzer archived pictures to allow electronic comparison of gel banding patterns.

Data Analyses

Measurements of shoot density, blades per shoot, shoot length, and shoot width were subjected to analysis with Principal Components Analysis (PCA) to detect relationships among these parameters and to general a composite variable to summarize

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variation in morphology (SAS 1999). The first principal component, which explained 96% of the variance in the data, consisted of five eigenvectors (Table 4). Each bed's mean morphotype (PC1 value) was subtracted from the mean morphotype of each other bed to generate morphological distances.

After scoring all gels, data for all loci were collapsed to generate a composite genotype for each plant. Composite genotype data were entered into Arlequin (Schneider *et al.* 2000), which was used to calculate indices of genetic diversity for beds and, when appropriate, for individuals. Equations for these indices can be found in Appendix I. The calculated indices included P, the percent of loci (of 7) that revealed polymorphisms (i.e., frequency of the most common allele < 99%); A, the mean number of alleles over all 7 loci; G, genotypic diversity within a bed; and H, observed heterozygosity (Endler 1986). Wright's (1978) F statistics were also calculated. F_{IS} is normally considered an estimate of the degree of inbreeding within a population, although in a clonal organism this value can be biased by multiple samplings of individual clones. The incidence of clone resampling was reduced by taking samples at least 2 m from one another. F_{ST} measured the amount of genetic subdivision among all beds. Arlequin (Schneider *et al.* 2000) was used to compare observed with expected heterozygosity and determine whether populations were in Hardy-Weinberg equilibrium.

Nested ANOVA (Zar 1998, SAS 1999) was used to examine the influence of age, size, and age/size combinations on each genetic diversity measure. In these ANOVAs, site (i.e., k = 4 individual beds per bed type) was nested within bed type (k = 3: old and large, old and small, young and small), with 100 replicate plants per individual bed. Bed

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type was treated as a fixed factor. Because data did not meet ANOVA assumptions for some genetic variables, resampling analyses were used to test for differences among bed types. For a given variable, the values for the 12 beds were sampled (with replacement) 10,000 times, and with each iteration, a mean value per bed type was calculated. The observed difference between the largest and smallest mean was then calculated, and this value was compared to that calculated from the bootstrapped replicates. The number of bootstrapped replicates whose value was greater than this difference was divided by the total number of bootstrapped replicates to generate a p-value.

An Arcview (2001) macro (Farnsworth 2001) was used to calculate distances between all sampling points. Data for Nei's (1972) genetic distances were generated by Arlequin. We used Mantel tests (Schneider *et al.* 2000) to correlate genetic and morphological distances among beds and genetic and geographic distance within and among beds.

RESULTS

Eelgrass from separate beds differed substantially in morphology (blade length, width, and total area; Figure 2) as well as in blades per shoot (Figure 3A) and shoot densities (Figure 3B). All of these measurements showed highly significant variation among beds ($p = 0.0001$ for all). Some phenotypic measures were correlated with one another (Table 5), but no morphological measure differed significantly among the three bed types (see Appendix II for details). Thus, there was no consistent effect of bed age or size on eelgrass morphology. While most correlations were weak, there was a stronger

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and highly significant positive correlation between blade length and width ($r^2 = 0.448$; $p < 0.0001$), indicating that long blades were generally also wide.

Overall genetic diversity of the eelgrass beds surveyed was high. In samples from nearly 1200 eelgrass individuals, a total of 73 composite (7-locus) genotypes were found. Of the seven polymorphic loci screened, 57 – 100% were polymorphic within all beds (Figure 4A), and mean allelic diversity (A) at a locus ranged from 1.625 – 2.000 over all beds and loci (Figure 4B). Genotype diversity (G) ranged from 0.12 to 0.40 (mean = 0.20) (Figure 4C). None of these genetic diversity measures varied significantly with bed age or size, although P tended to be lower in old, small beds (resampling analyses; $p = 0.0567$). There was substantial variation among individual beds in P, A, and especially G. Heterozygosity ranged from 0.21 to 0.87 (Figure 5A). All beds deviated from Hardy-Weinberg equilibrium, with significant heterozygote deficiencies (ANOVA; $p = 0.0002$) (Figure 5B).

F_{IS} was variable among beds, ranging from 0 to 0.91 (mean = 0.68) (Figure 5C). Resampling tests ($n = 10{,}000$) showed that this inbreeding coefficient differed significantly among the three bed types ($p = 0.0321$), as old, small beds were less inbred than other bed types. F_{ST} over all beds was 0.1976, a high level of genetic substructuring (Wright 1978).

There was no relationship between genetic and morphological distance either among (Figure 6; $r^2 = 0.0000$, $p = 0.9612$) or within ($r^2 = 0.0000$, $p = 0.7582$; data not shown) beds. Mantel tests showed no relationship between Nei's (1972) genetic distance and geographic distance among beds (Figure 7; $r^2 = 0.0559$, $p = 0.0541$), and this was

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reflected in the distribution of composite genotypes (Figure 8). Finally, there was no relationship between Nei's (1972) genetic distance and geographic distance within any bed (Mantel tests; for each bed, $p \geq 0.065$).

Tables of all statistical analyses can be found in Appendix II.

DISCUSSION

Genetic diversity values reported in this study of Chesapeake Bay eelgrass were intermediate compared to those reported for other plant species (Hamrick 1983, Jelinski 1997, Francisco-Ortega *et al.* 2000, van der Bank *et al.* 2001) and were higher than most reported in Williams and Orth's (1998) survey of Chesapeake Bay *Z. marina*. There are several possible explanations for the differences between this and the previous eelgrass data set. The most likely is that this data set used 7 polymorphic loci while Williams and Orth used 12 loci, three of which were monomorphic; this would have reduced the estimate of genetic diversity averaged across all loci.

Although morphology varied strongly among beds, no morphological measure varied consistently with bed age or size. Oostermeijer *et al.* (1994) suggested that founder effects should create more genetic and phenotypic variability among small populations than large, which seems to agree with our data (Figures 4C, 5A), although trends did not approach statistical significance. This reinforces the conclusion from earlier transplant experiments (Rhode 2002; Chapter 4) that morphological variation in Chesapeake Bay eelgrass is affected more by environmental than genetic factors and supports the current study's finding of no relationship between genetic and

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morphological distance.

Measures of genetic diversity also did not vary consistently with bed type. Neither the amount of time over which a bed could have experienced selection (> 65 years vs. < 7 years) nor bed size affected genetic diversity. Several explanations for this result are possible. First, the difference in age might be too small to observe appreciable selection effects. Alternatively, bed age estimates could be misleading. Although this study benefited from accurate estimates of bed persistence (presence of eelgrass at a particular spot) beds might not be discrete populations. If population immigration or emigration is significant, or if clonal propagation prolongs the genetic life of short-lived individuals, areal coverage estimates of population age might be gross overestimates.

Larger beds did not have more genetic diversity than their smaller counterparts, perhaps because their large size can be attributed to clonal growth rather than seed germination. Alternatively, perhaps the genetic diversity of small beds is relatively high because they are founded by multiple clones. This is consistent with the observation that seeds are transported as sibling clusters attached to maternal reproductive shoots.

F_{ST} values in this study were consistent with those reported previously (Ruckelshaus 1998, Williams and Orth 1998), indicating "great" amounts of genetic differentiation among beds (Wright's scale of comparison; Wright 1978). This means that dispersal among beds followed by successful reproduction (i.e., gene flow) occurs over only limited distances, that such successful migration is rare, or that migration that does occur is obscured by high levels of clonal growth and inbreeding within beds.

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Along with displaying strong genetic differentiation among beds, individual beds showed significant heterozygote deficiencies. These deficiencies are probably attributable in part to Wahlund effects, an apparent reduction in genetic diversity that is, in fact, a consequence of sampling multiple genetic populations and analyzing them as if they are a single population. Since dispersal of eelgrass gametes and seeds is somewhat limited, it is likely that beds (used here as units of population structure) were in fact mosaics of locally interbreeding groups of plants. Heterozygote deficiencies could also result from inbreeding. F_{IS} indicated that inbreeding was substantial in all beds, although inbreeding in old, small beds was significantly lower than in other bed types. The latter result is somewhat puzzling but is consistent with Ruckelshaus' (1998) finding that small beds had significantly more inbreeding than large beds. Mean F_{IS} values reported in this study were higher than those reported in Williams and Orth (1998) (0.680 vs. 0.144). It is rare for selection to act against heterozygotes (Endler 1986), so observed heterozygote deficiencies were probably due to vegetative reproduction or to non-random mating in the form of self-fertilization, or inbreeding (including gamete exchange among clonemates). This is consistent with evidence that inbreeding occurs *in situ* with some regularity in eelgrass (Ruckelshaus 1996) and that Chesapeake eelgrass is adapted to selfing (Rhode 2002, Chapter 3). Inbreeding might be further reinforced by pollen-dispersal distances of less than 15 m (deCock 1980; Cox *et al.* 1992; Ruckelshaus 1994, 1996), a range not broad enough to cover the unvegetated waters between beds (Williams and Orth 1998, Reusch *et al.* 1999). This seems reasonable, as there was no relationship between Nei's

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genetic distance and geographic distance, in spite of the fact that some beds surveyed were quite close (less than 5 km apart).

Genetic structure documented here suggested several conclusions about the history of Chesapeake Bay eelgrass. Overall eelgrass diversity showed no evidence of being impacted by demographic bottlenecks. This implies that the two strong population reductions experienced by Chesapeake eelgrass beds during the last century were not severe enough to cause a drastic reduction in genetic variance. Alternatively, it is conceivable that genetic diversity could have been supplemented by substantial immigration of seeds or shoots from nearby beds. Finally, perhaps the observed genetic diversity is still only a fraction of pre-1930's levels. Data from this chapter combined with those in Chapter 4 (Rhode 2002) show no correlation between morphology or genetics and fitness; this supports the hypothesis that new eelgrass beds are established and maintained by non-selective demographic processes (Reusch 2002).

Genetic diversity patterns also reveal something about processes currently happening in eelgrass beds. There was no relationship between genetic and morphological distance either bay-wide or within beds, perhaps due to phenotypic plasticity (Rhode 2002; Chapter 4). The underlying plasticity of eelgrass allows successful response to variable or novel environments, and it, rather than genetic differences, could be adaptive. In fact, recent literature has argued that phenotypic plasticity is crucial to the survival and evolution of species and is particularly important to plants (Sultan 2000, Agrawal 2001).

This study found no local adaptation (Rhode 2002, Chapter 4) and no evidence

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that genetically depauperate beds experienced negative fitness consequences. This further supports the hypothesis that eelgrass genetic structure is the result of low gene flow (Ruckelshaus 1996) and little successful, long-term seedling recruitment (Ewanchuk 1995, Hootsmans *et al.* 1987). Mantel tests showed no relationship between genetic and geographic distance among beds, perhaps because realized dispersal distances were not long enough to create connections between beds. Alternatively, the patchy beds surveyed could be genetic remnants of what was once a single continuous population. Finally, the distance measurements used in the Mantel tests could be misleading, as currents do not always follow paths of shortest distance.

Genetic diversity patterns can also be used to predict or make recommendations about a population's future. Since evidence of local adaptation in these populations is weak (Rhode 2002; Chapter 4), preserving total bed coverage might take precedence over conservation of genetic diversity. This is a risky proposition, though, as it is impossible to predict the effects of genetic diversity on eelgrass response to future stresses, and a previous study showed a correlation between population genetic diversity and bed growth (Williams 2001). Sustaining genetic diversity can be crucial in maintaining the adaptive potential and resilience of populations of most species, including seagrasses (Ruckelshaus 1994; McRoy 1996; Williams and Orth 1998, Procaccini and Piazzi 2001, Williams 2001).

Because much genetic diversity is divided among high-diversity beds, the source from which transplanted material is taken can greatly affect the genetic structure of the created population. The data in this chapter and in Williams and Orth (1998) reveal

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many genetic differences among eelgrass beds, so maintaining maximal genetic diversity of Chesapeake Bay eelgrass means minimizing destruction of whole beds. Data presented here suggest that the ideal size and diversity of restored beds could vary, and that small or young beds are not necessarily depleted in genetic resources. Instead of choosing source beds based on their size, age, or genetic diversity, it is probably acceptable to choose beds according to convenience (i.e., proximity of donor bed to transplant site, bed depth, etc.) Indeed, this strategy was employed in all eelgrass restoration efforts in Chesapeake Bay which, until recently, relied on a single donor bed for transplant material. The genetic data presented here offer no evidence against this strategy, although the longer-term effects of genetic homogeneity on these beds remain unknown.

Other studies have suggested that, in general, more genetically diverse populations have greater fitness (Oostermeijer *et al.* 1994, Williams 2001). The data reported here and in Chapter 4 (Rhode 2002) offer no clear support for such a relationship in Chesapeake Bay eelgrass. This study did not measure performance directly or over time, however. Although a single multilocus genotype dominated most surveyed beds (Figure 8), eelgrass in these places seems to flourish. Shoot length, width, and area, all good indicators of eelgrass performance, were not related to genetic diversity measurements. This suggests that phenotypic plasticity might be able to offset lack of genetic diversity in this species. Results of a reciprocal transplant study also indicate that phenotypic plasticity is the mechanism by which eelgrass plants in Chesapeake Bay adapt to novel environments (Rhode 2002, Chapter 4). Linhart and

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Grant (1996) asserted that most genetic substructure in plant populations results from natural selection rather than non-selective processes. The lack of local adaptation and plethora of phenotypic plasticity make it likely that Chesapeake Bay eelgrass is one exception to this generalization.

Resource managers have operated under the simplifying assumption that seagrasses spread primarily through clonal growth; hence, re-vegetation of decimated beds is currently done with no attention to their genetic composition. While the underlying reproductive assumption is probably incorrect (Rhode, Chapter 3 and this chapter), the fitness consequences for created beds seem identical. It is important to note, however, that this study did not measure long-term performance of eelgrass in these beds, only aspects of genetic structure. In contrast, Williams (2001) measured both performance and genetic characteristics of transplanted beds. Her data showed that eelgrass transplants had substantially reduced genetic diversity, probably because material collected for transplantation was not collected bed-wide. This reduction in diversity was correlated with decreased rates of bed growth and reductions in individual fitness. Thus, a precautionary principal would suggest that maintaining genetic diversity and consequent potential for bed growth and response to environmental change would be desirable in Chesapeake Bay eelgrass as well. With Williams' data in mind, future studies should monitor populations over time to look for correlations between genetics and performance or to track changes in genetic makeup.

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Table 1. Age and size of *Zostera marina* beds used in genetic survey. Areal coverage values were obtained from 1997 survey data (Orth *et al.* 1998). For maps with multiple beds, the bed number (Orth *et al.* 1998) is indicated in parentheses.

eelgrass bed	**age (y)**	**areal coverage (ha)**	**bed type**
Allen's Island	>65	141.30	old, large
Brown's Bay	>65	159.81	old, large
Poquoson Flats	>65	207.92	old, large
Tangier Island	>65	212.92	old, large
Broad Bay (map A2)	>65	1.10	old, small
Gwynn's Island	>65	5.47	old, small
James River	>65	7.68	old, small
Milford Haven (map T2)	>65	0.67	old, small
Fisherman's Island	6	0.40	young, small
James River (map E1)	<2	4.16	young, small
Little Creek	<5	3.77	young, small
Yorktown	6	8.20	young, small

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Table 2. Allozyme systems screened and results of screening. 0 = not scorable; A = activity, but not scorable consistently; S = scorable. Systems were tested as in Soltis *et al.* (1983), Richardson *et al.* (1986), Murphy *et al.* (1986), and Williams and Orth (1998).

Buffer	**system**	**result**
MC	AAT	A
LiB	ACP	A
MC	ADH	S
TC	ADH	S
LiB	ADH	A
LiB	ACP	A
LiB	CAT	A
MC	CAT	0
TEB	EST	A
MC	EST	0
LiB	FE	0
TEB	G6PDH	A
LiB	GOT-1	A
LiB	GOT-2	A
MC	GPI-1	S
MC	GPI-2	S
TEB	GPI-1	A
TEB	GPI-2	A
MC	IDH	S
TC	IDH	S
MC	LDH	A
TEB	LDH	0
MC	MDH-1	S
MC	MDH-2	S
TC	MDH-1	A
TC	MDH-2	A
MC	ME	S
MC	ME-2	A
LiB	PGM	A
MC	PGM	A
MC	PRX	0
MC	SOD	A
TEB	TPI-1	0
TEB	TPI-2	0

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Table 3. Allozyme systems used in Williams and Orth (1998) and in this study. Systems used in both are italicized. Enzyme and buffer abbreviations follow Enzyme Commission convention.

Williams and Orth (1998)		this study	
buffer system	**stain**	**buffer system**	**stain**
LiB	CAT	TC	ADH
LiB	FE	*MC*	*GPI-1*
TEB	G6PDH	*MC*	*GPI-2*
LiB	GOT-1	*MC*	*IDH*
LiB	GOT-2	*MC*	*MDH-1*
MC	*GPI-1*	*MC*	MDH-3
MC	*GPI-2*	MC	ME
MC	*IDH*		
MC	*MDH-1*		
LiB	PGM		
MC	PRX		
TEB	TPI-1		
TEB	TPI-2		

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Table 4. The five eigenvectors which comprised the first principal component in PCA analysis of eelgrass morphology. This principal component explained 96% of the variance in the data.

factor	**multiplier**
0.0128	density
0.7777	length
0.0204	width
0.6281	blade area
0.0018	blade number

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Table 5. Pearson correlations among eelgrass morphological measures. In each cell, top number is value of the correlation (r^2), and bottom number is the p value. Values not accounted for by correlations can be attributed to error. P values ≤ 0.05 are in bold. n = 1026 for each measure (not 1200; some morphometric data missing).

	shoot density	blade length	blade width	blades / shoot
shoot density	1			
blade length	0.081 **0.009**	1		
blade width	0.098 **0.002**	0.448 **0.000**	1	
blades / shoot	0.075 **0.016**	-0.085 **0.006**	0.102 **0.001**	1

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Figure Legends

Figure 1. Map of Chesapeake Bay (Virginia, USA) indicating locations of beds surveyed for this study. Old, large beds are labeled with white letters; old, small beds are labeled with grey letters; young, small beds are labeled with black letters. These colors correspond with those used in other figures.

Figure 2. Length, width, and blade area of eelgrass in 12 Chesapeake Bay beds. n = 100 per bed. There were significant differences in blade length, width, and area among the 12 beds (1-way ANOVAs, p = 0.0001 for each).

Figure 3. Number of blades per shoot and shoot density of eelgrass in 12 Chesapeake Bay beds. n = 100 per bed. There were significant differences in blades per shoot and areal coverage among beds (1-way ANOVAs, p = 0.0001 for each). ND = no data available.

Figure 4. Measurements of genetic diversity for 12 Chesapeake Bay eelgrass beds. A) P, percent loci polymorphic, B) A, average allelic diversity , and C) G, proportion distinct genotypes. Values were based on composite genotypes (7 allozyme loci); n = 100 plants per bed. Resampling analysis (n = 10,000) showed no differences in P (p = 0.057), A (p = 0.275), or G (p = 0.614) among bed types.

Figure 5. Measurements of heterozygosity and inbreeding for 12 Chesapeake Bay eelgrass beds. A) mean (±1 standard error) H, proportion heterozygous individuals (of n = 100) within bed, over 7 loci, B) deviation from expected heterozygosity for each bed, and C) F_{IS} , inbreeding coefficient, for each bed. Measurements of $H_O - H_E$, and F_{IS} were based on composite genotypes (7 allozyme loci) of 100 plants per bed. F_{IS} differed significantly among bed types (resampling analysis; n = 10,000; p = 0.032).

Figure 6. Relationship between morphological distance (determined by PCA analysis) and Nei's genetic distance among 12 Chesapeake Bay eelgrass beds. Values are based on measurements of n =100 plants per bed.

Figure 7. Relationship between Nei's genetic distance and geographic distance among 12 Chesapeake Bay eelgrass beds. Values are based on composite genotypes (7 allozyme loci) of n = 100 plants per bed.

Figure 8. Distribution of composite genotypes (7 allozyme loci) among and within Chesapeake Bay eelgrass beds. Each color represents a distinct composite genotype. n = 100 plants per bed.

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Figure 1

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Figure 2

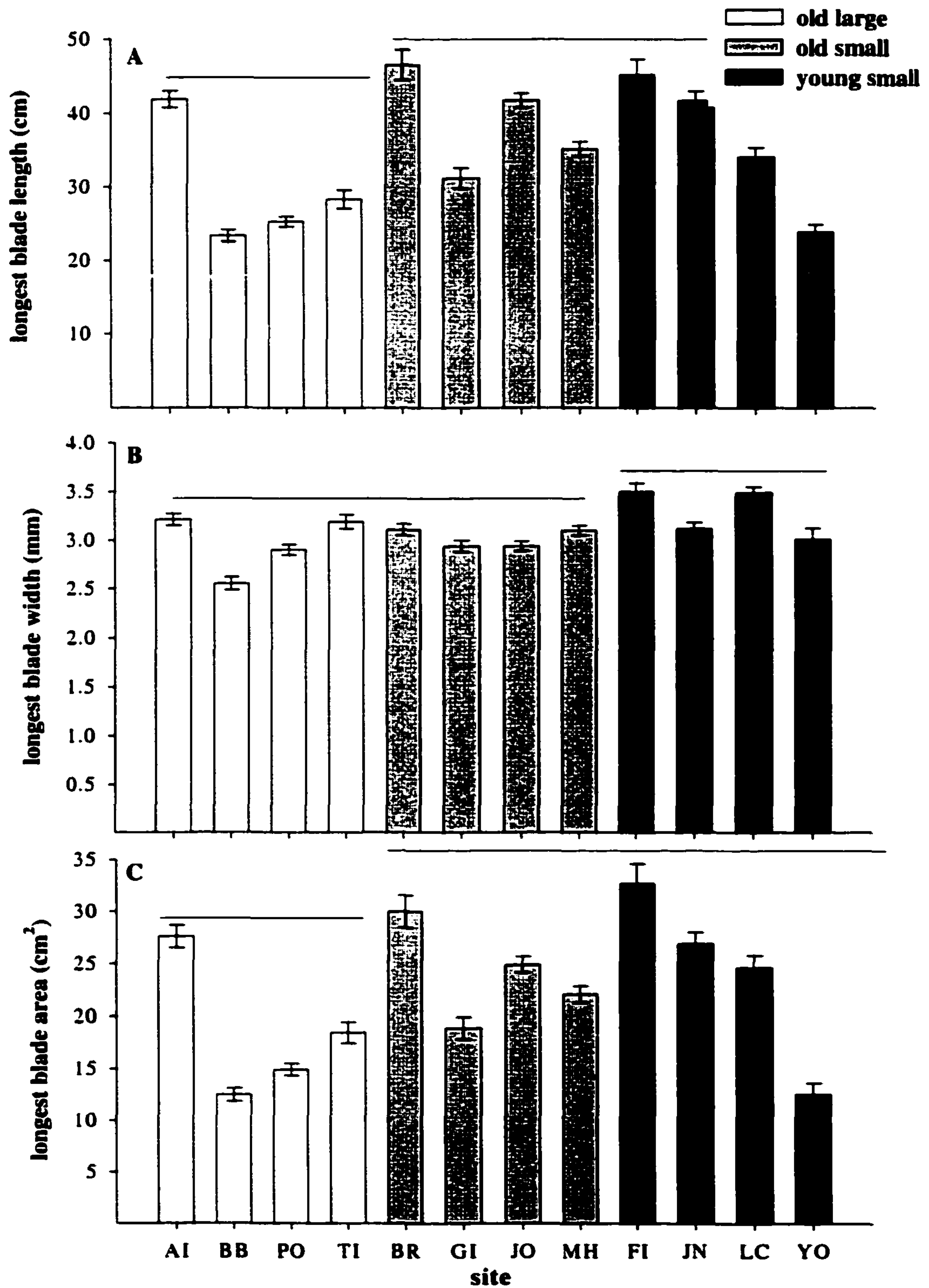

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Figure 3

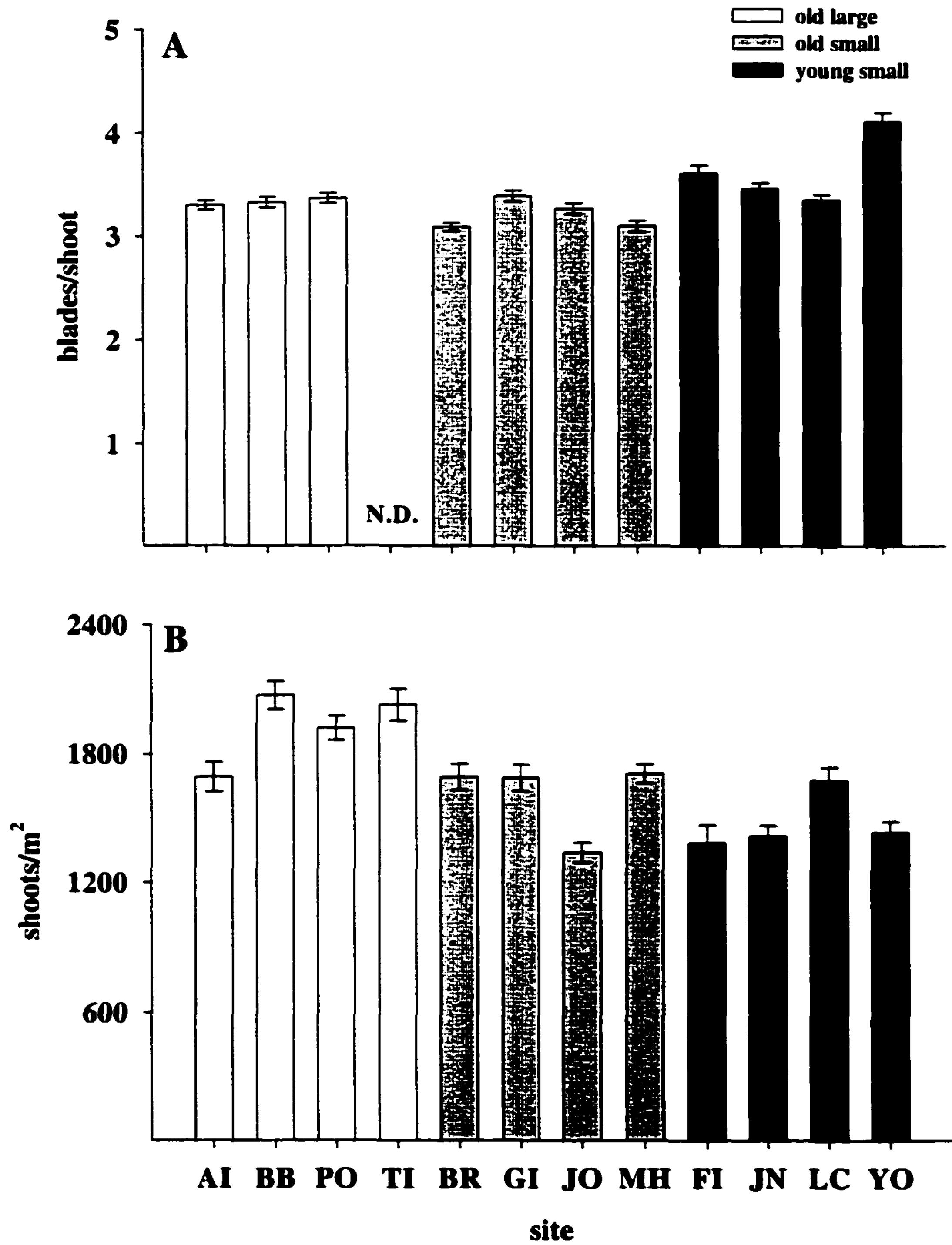

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Figure 4

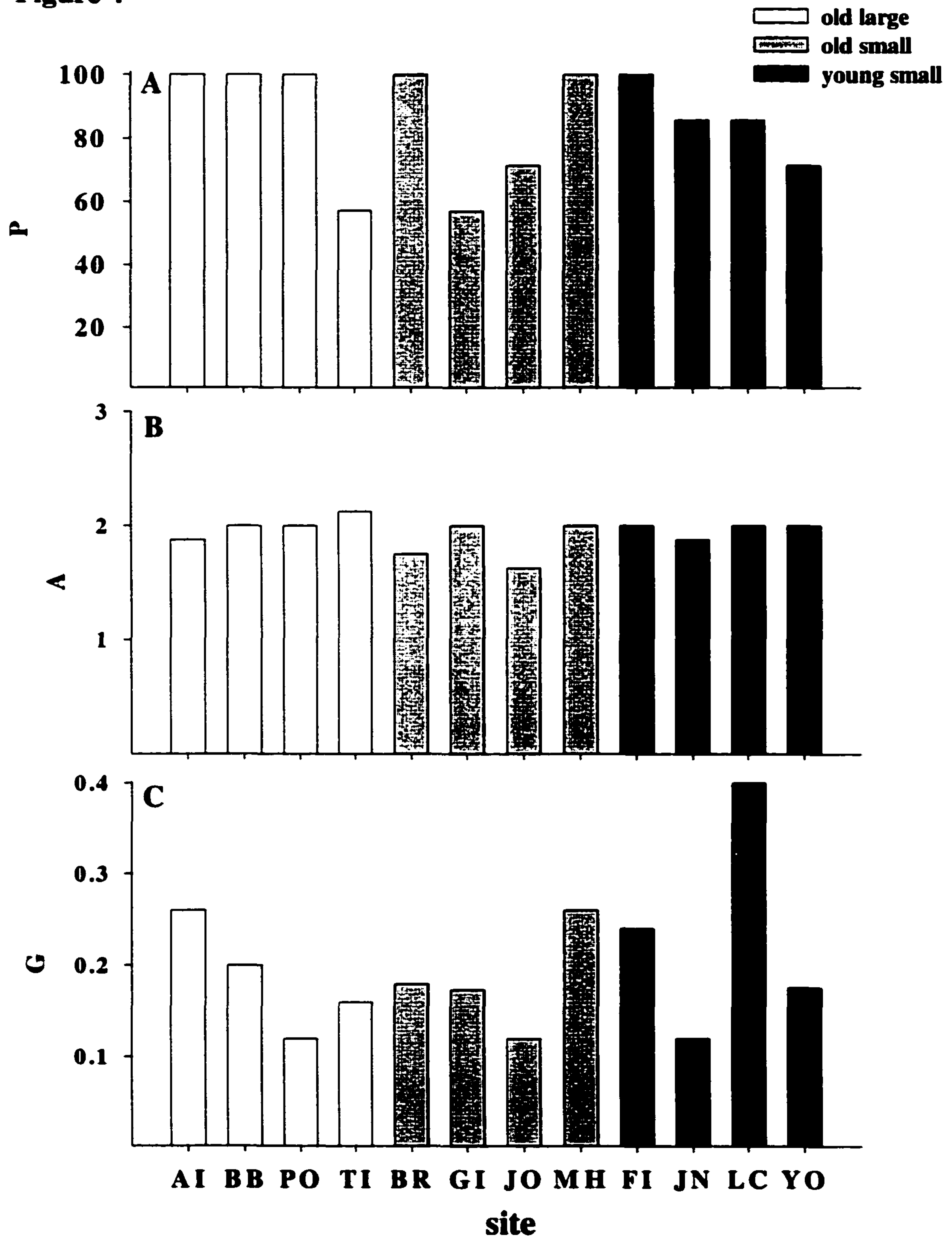

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Figure 5

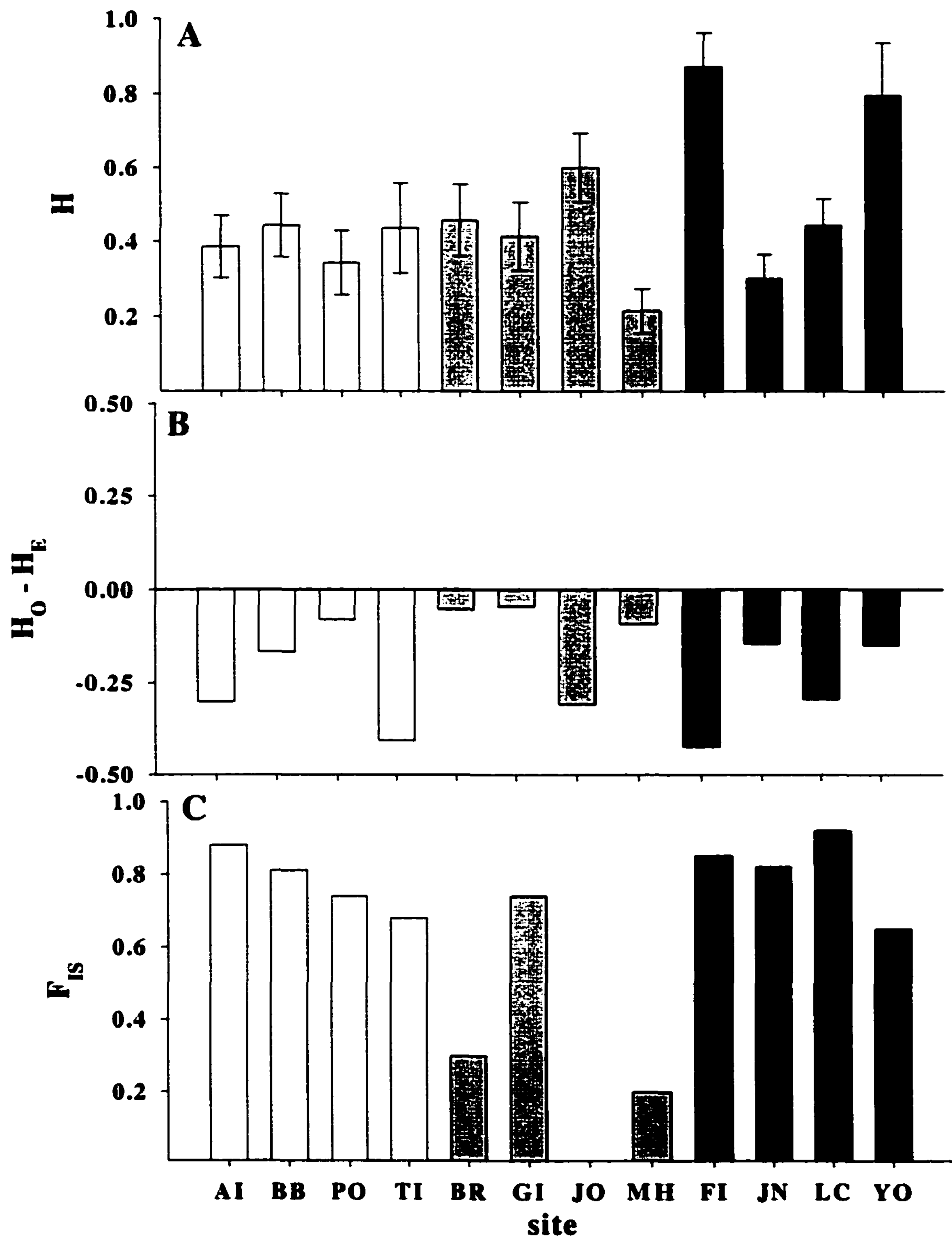

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Figure 6

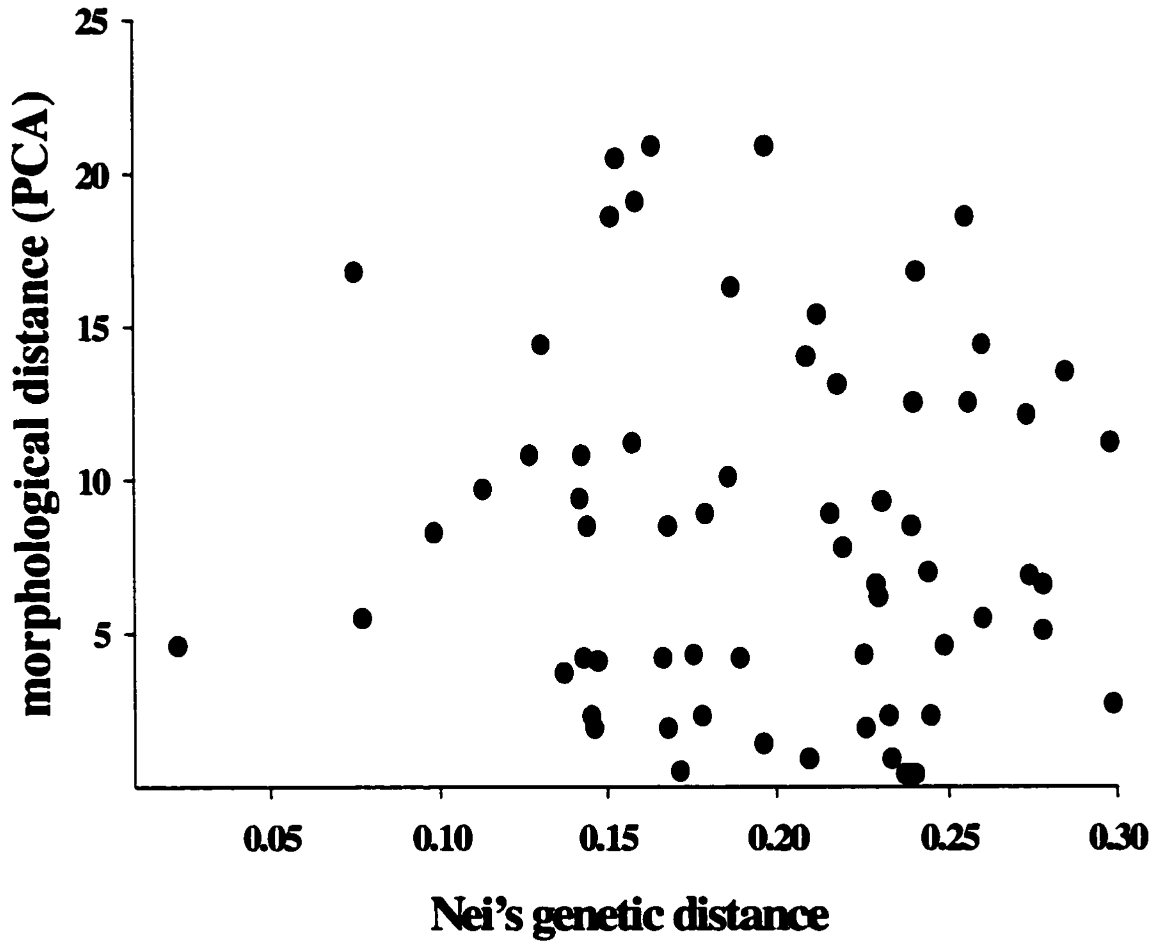

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Figure 7

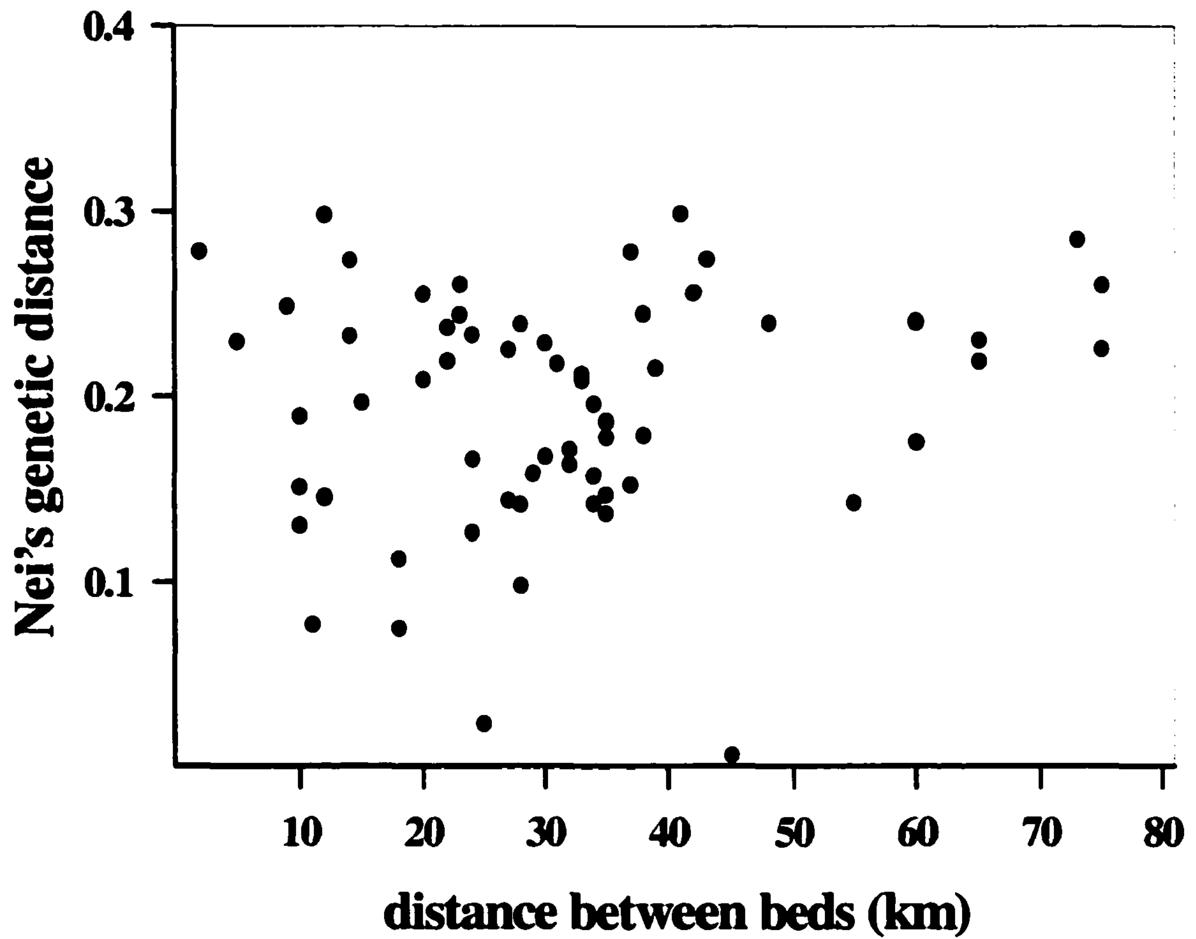

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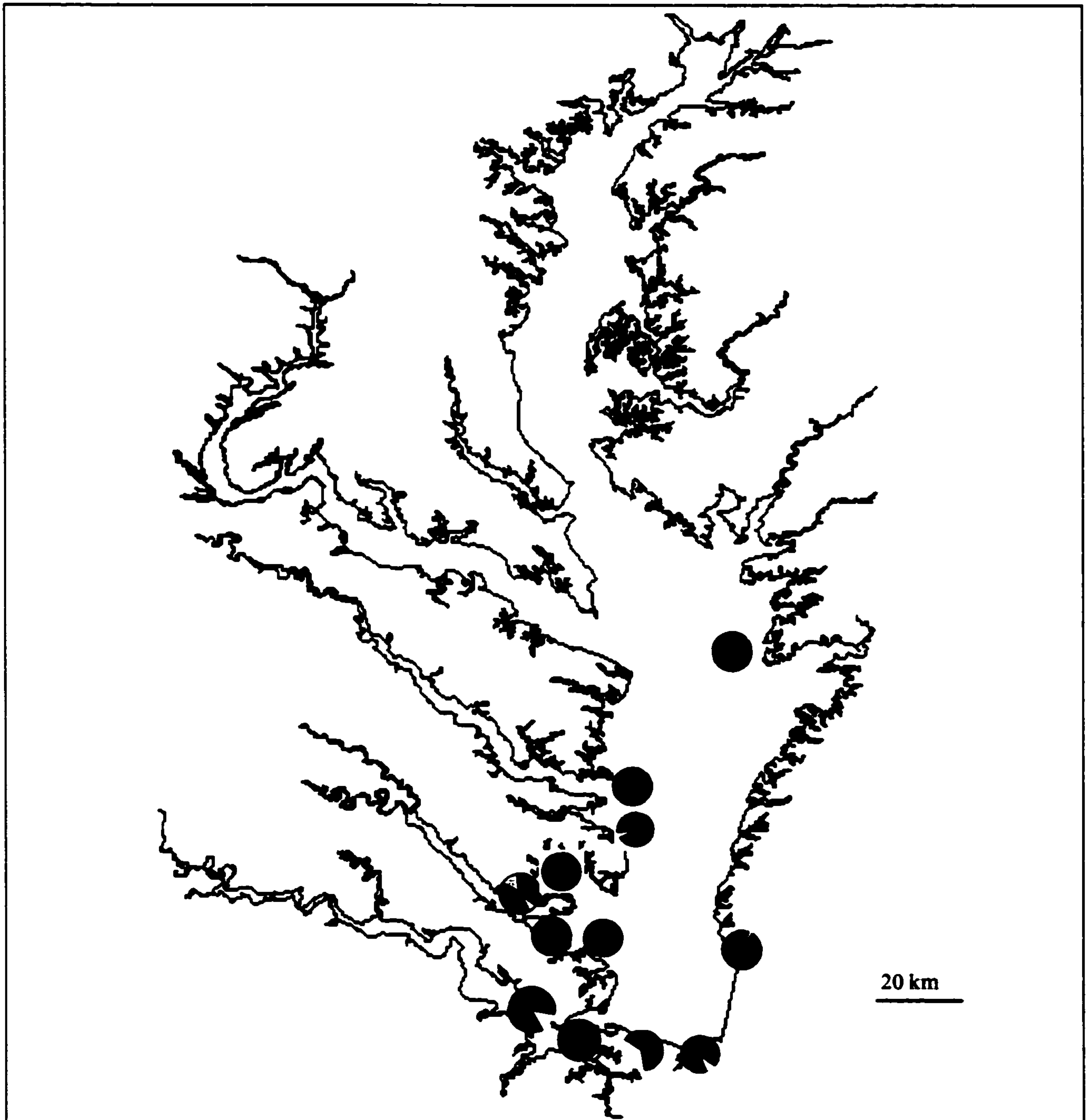
Figure 8



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Appendix I: Equations for Measures of Genetic Diversity

Formulae are modified from Avise (1994).

$$P_{NC} = \frac{\left(\sum \text{polymorphic loci}\right)}{\left(\sum \text{total loci}\right)}$$

$$A = \frac{\left(\sum \#\ \text{alleles}\right)}{\left(\sum \text{total loci}\right)}$$

$$G = \frac{\left(\sum \#\ \text{unique genotypes}\right)}{\left(\sum \text{total individuals}\right)}$$

$$H_o = \frac{\left(\sum \#\ \text{heterozygous loci}\right)}{\left(\sum \#\ \text{loci}\right)}$$

$$N_M = \frac{(1/F_{ST} - 1)}{4}$$

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Appendix II: Statistical Tables

Nested ANOVA: Blade Length as a Function of Bed Type and Site

	sum of squares	*mean square*	*F*	*p*
bed type	17548.2763	8774.1381	1.41	0.2939
site (bed type)	56106.6557	6234.0729	41.29	<0.0001
error	169590.9037	150.6136		

Nested ANOVA: Blade Width as a Function of Bed Type and Site

	sum of squares	*mean square*	*F*	*p*
bed type	19.1682	9.5841	1.85	0.2118
site (bed type)	46.5335	5.1704	12.46	<0.0001
error	467.2950	0.4150		

Nested ANOVA: Blade Area as a Function of Bed Type and Site

	sum of squares	*mean square*	*F*	*p*
bed type	9783.4691	4891.7346	1.44	0.2869
site (bed type)	30591.4744	3399.0527	31.31	<0.0001
error	1.22270.4866	108.5884		

Nested ANOVA: Blade Number as a Function of Bed Type and Site

	sum of squares	*mean square*	*F*	*P*
bed type	22.7773	11.3887	2.51	0.1356
site (bed type)	40.7569	4.5285	15.73	<0.0001
error	291.9716	0.2879		

Nested ANOVA: Shoot Density as a Function of Bed Type and Site

	sum of squares	*mean square*	*F*	*P*
bed type	418.6173	209.3086	0.70	0.5236
site (bed type)	2707.0772	300.7864	97.84	<0.0001
Error	3519.9787	3.0742		

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Resampled Genetic Diversity Indices

	resampled mean			
	old (O)	*new (N)*	*small (S)*	*large (L)*
Ho	0.086	0.096	0.084	0.100
P	73.438	71.875	78.125	62.500
A	1.953	2.031	1.969	2.000
G	0.218	0.165	0.205	0.190

	largest – smallest mean	*P (3 bed types)*	*p: large vs small*	*p: young vs. old*
Ho	0.02755	0.3165	0.6100	0.3228
P	21.87500	0.0567	0.2757	0.1401
A	0.12500	0.2747	0.2722	0.6207
G	0.08000	0.6137	0.6133	0.2925

Resampled F_{IS}

old small	*mean*	0.654
old small	*SEM*	0.120
old small	*variance*	0.057
old large	*mean*	0.518
old large	*SEM*	0.157
old large	*variance*	0.099

largest - smallest mean: 0.224
p = 0.0321

Principal Components Analysis: Morphological Characters

covariance matrix

	density	*length*	*width*	*area*	*blade number*
Density	5.9910	2.9448	0.1662	3.3298	0.1099
Length	2.9448	216.0206	4.4930	165.0507	-0.7458
Width	0.1662	4.4930	0.4692	5.7730	0.0414
Area	3.3298	165.0507	5.7730	144.8667	-0.0734
blade number	0.1099	-0.7458	0.0414	-0.0734	0.3484

total variance = 367.6958

eigenvalues of the covariance matrix

	Eigenvalue	*difference*	*proportion*	*cumulative*
1	349.4876	337.5359	0.9505	0.9505

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2	11.9517	6.1079	0.0325	0.9830
3	5.8438	5.5128	0.0159	0.9989

eigenvectors

	pc1	*pc2*	*pc3*
Density	0.0128	0.1233	0.9921
Length	0.7777	-0.06201	0.0670
Width	0.0204	0.1418	-0.0232
Area	0.6281	0.7608	-0.1024
blade number	0.0018	0.0367	0.0119

Regression: Morphological and Geographic Distance

	df	*sum of squares*	*mean square*	*F*	*p*
morphology/geography	1	11.5641	11.5641	0.34	0.5622
Error	64	2179.9732	36.0621		

$r^2 = 0.0053$

Regression: Morphological and Genetic Distance

	df	*sum of squares*	*mean square*	*F*	*p*
morphology/genetic	1	0.0815	0.0815	0.00	0.9612
Error	64	2191.4558	34.2415		

$r^2 = 0.0000$

Regression: Geographic and Genetic Distance

	df	*sum of squares*	*mean square*	*F*	*p*
geography/genetics	1	0.01612	0.01612	3.85	0.0541
Error	64	0.2723	0.00419		

$r^2 = 0.0559$

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Chapter 3: Reproductive Strategies of Chesapeake Bay (Virginia, USA) Eelgrass, *Zostera marina* L

ABSTRACT

Plant mating strategies have important effects on population demography and fitness. In monoecious plants, gametes can be exchanged across populations (outbreeding), with close relatives (inbreeding), or within individuals (selfing). Inbreeding or selfing are expected when access to mates is limited, and under some conditions of environmental stress. Highly limited pollen dispersal and strong population subdivision in Chesapeake Bay eelgrass suggest conditions favorable to inbreeding or selfing. However, eelgrass flowering is asynchronous, with females emerging first. Because inbreeding depression is common in many organisms, non-selfed matings were predicted to be most successful. The relative fitness of outbreeding, inbreeding, and self-fertilization in three Chesapeake Bay populations of *Zostera marina* L (eelgrass) was examined by hand-fertilizing flowers and monitoring fertilization success and seed production. Selfed matings produced seeds significantly more frequently than outcrossed matings and produced significantly larger numbers of seeds than either inbred or outbred matings. Though genetic data showed widespread inbreeding in Chesapeake Bay eelgrass, it is unlikely that this inbreeding has negative consequences for fitness, since results of the mating experiments showed no evidence of inbreeding depression and indeed indicate that selfing has the highest fitness.

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These results are consistent with expectations for populations with limited gamete migration and strong small-scale genetic structure, as previously demonstrated in Chesapeake Bay eelgrass.

keywords: *Zostera marina*, dichogamy, mating system, inbreeding, outbreeding

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INTRODUCTION

Sexual reproduction in plants can occur among populations, within populations, or even within individuals. Consequences of mating patterns for the genetic and demographic structure of populations and for individual fitness can be substantial (Waser 1993a). For example, repeated inbreeding can result in loss of heterozygosity, leaving populations vulnerable to extinction in the face of shifting environmental conditions. As a result of both intrinsic and external constraints, different plant taxa display a continuum of mating strategies, from inbreeding, including selfing, to outcrossing.

Inbred matings occur between individuals from a single population who share alleles by descent (Waser 1993b). Populations that are sessile, are physically fragmented, or have limited pollen and seed dispersal distances often inbreed (Waser 1993a). These consanguineous matings can increase each parent's genetic representation in the next generation, preserve coadapted gene complexes, maintain local adaptation, and, in contrast to asexual propagation, provide a mechanism for mutational purging (Waller 1993). Conversely, inbreeding reduces heterozygosity and increases the expression of deleterious recessive alleles within a population. Resultant decreases in offspring fitness are termed inbreeding depression and have been detected in several terrestrial plant species (reviewed in Waser 1993a). Models predict that inbreeding depression, however, can be overwhelmed by strong local adaptation (Wiener and Feldman 1993). If populations occupy distinctly different environments to which they become locally adapted, outbreeding can reduce fitness by introducing foreign genotypes poorly adapted

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to local conditions. In such situations inbreeding can maximize fitness (Waser and Price 1989, Schmitt and Gamble 1990).

Self-fertilization is an extreme form of inbreeding in which gametes from a single individual fuse. This phenomenon maximizes the parent's genetic contribution to its offspring, avoiding recombination with non-adapted genotypes; it provides reproductive assurance while retaining a mechanism for outbreeding (Waser 1993b). Plant species that self-fertilize regularly are usually either annuals or biennials whose temporal window for reproductive success might be limited (Aarssen 2000). In heterozygous individuals, self-fertilization can generate some genetic variability. However, offspring produced by selfed matings often experience inbreeding depression, manifested as accumulation of deleterious mutations and reduced heterozygosity. To avoid these negative effects, many plants have evolved pre-mating barriers to self-fertilization, including asynchronous flowering or receptivity, or mechanical mismatches, and post-mating barriers, such as gametic incompatibilties (Charlesworth and Charlesworth 1987). The frequency of self-fertilization is affected by life history characteristics, including the chronology of gamete maturation.

Outbred matings occur between individuals who come from different populations and are not related (Waser 1993b). Outbreeding tends to homogenize population genetic structure and can increase genetic diversity. Conversely, the subsequent break up of multi-locus genotypes and potential disruption of local adaptation can result in reduced offspring fitness, or outbreeding depression (e.g. Montalvo and Ellstrand 2001). This

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phenomenon has been experimentally demonstrated via artificial matings of several terrestrial plant species but seems to occur less frequently than inbreeding depression (reviewed in Waller 1993 and Waser 1993a).

This work studied the mating system of eelgrass, the northern hemisphere's most widespread and abundant temperate marine angiosperm. *Zostera marina* is monoecious, with female and male flowers on a single inflorescence (Figure 1), so genetic exchange might occur within and between local individuals as well as between populations. *Zostera marina* produces flowers in its second year and seems to be a biennial (Setchell 1929, Harwell 2000). Flowering shoots and the inflorescences they bear mature acropetally, from the base toward the tips (de Cock 1980). Flowers on a single plant emerge asynchronously, with stigmas maturing first (protogyny) and pollen released 48 hours later. Thus, self-fertilization within eelgrass inflorescences is probably rare in nature, though geitonogamy (self-fertilization among different infloresences) might occur. Vegetative expansion of eelgrass patches is rapid (Olesen and Sand-Jensen 1994a, b), seed production is unpredictable in both space and time (Silberhorn *et al.* 1983, van Lent and Verschuure 1995, Meling-Lopez and Ibarra-Obando 1999), and mortality of eelgrass seeds (Fishman and Orth 1996) and seedlings (Hootsmans *et al.* 1987) can exceed 90%.

Early allozyme surveys found little genetic diversity within or among eelgrass beds (Gagnon *et al.* 1980, McMillan 1982, Heij and Nienhuis 1992). Demographic and genetic data led some researchers to conclude that sexual reproduction contributes little to

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the establishment or maintenance of eelgrass populations (Les 1988). Recent surveys with additional loci or more powerful neutral markers (e.g. microsatellites) revealed more variation, however (Williams and Orth 1998; Reusch 2001; Rhode 2002, Chapter 2). Mating patterns could contribute to these observed differences among eelgrass beds.

Because eelgrass flowering tends towards protogyny, previous research assumed that most seeds were products of non-selfed, outbred matings (Setchell 1929, de Cock 1980, Phillips *et al.* 1983). Genetic and breeding studies done in Europe and North America support both the potential for (hand-pollination made self-fertilization possible) and rarity of (little seed production from selfed matings) self-fertilization in this species (Cox *et al.* 1992, Ruckelshaus 1995, Reusch 2000, Reusch 2001). Genetic and demographic studies have also shown that, though beds are often inbred, the relative fitness of offspring produced by geitonogamy (self-fertilization; matings between different flowers on a single plant) or inbreeding is low, so selfing results in inbreeding depression (Ruckelshaus 1995; Reusch 2000, 2001).

Eelgrass occupies broad ecological niches over vast geographic areas (Phillips *et al.* 1983), so the applicability of Ruckelshaus' (1995) and Reusch's (2000, 2001) studies to other eelgrass populations is unknown. Mating systems could be influenced by demographic constraints such as limited pollen and seed dispersal or historic fragmentation and patch demography; these differ widely across geographic regions (Harwell 2000, Reusch 2001). For example, if populations are locally adapted, they might be able to inbreed without adverse fitness consequences. It is even possible that

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genetically structured *Z. marina* populations, such as those in Chesapeake Bay (Williams and Orth 1998), experience outbreeding depression, although a recent study provides little evidence that populations are locally adapted (Rhode 2002, Chapter 4).

In a previous study (Rhode 2002, Chapter 2), F-statistics were used to characterize genetic structure of Chesapeake Bay eelgrass beds. As a follow-up, this paper describes artificial breeding experiments to determine the fitness consequences of different mating patterns for these eelgrass populations. Few seeds were expected to result from self-fertilization in this protogynous plant. Since beds are strongly genetically differentiated, though, outbreeding between individuals from distant populations was not expected to produce many seeds. Within-bed matings, in which seeds were sired by and developed on plants from a single bed, were hypothesized to produce the most seeds.

METHODS

Greenhouse

To determine the relative fitness of different types of mating in Chesapeake Bay eelgrass, controlled laboratory matings were conducted among individuals from three beds that are geographically, morphologically and genetically different (Rhode 2002, Chapter 2): Allen's Island, Brown's Bay, and Broad Bay (Figure 2) in Chesapeake Bay (Virginia, USA; 37° N, 76° W). In spring (April) 1998, 160 reproductive shoots were collected at haphazard locations within each of the three sites. Each reproductive shoot had an attached vegetative shoot to provide a source of photosynthate to its developing

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flowers and seeds. Distance between collection spots exceeded 2 m (as in Ruckelshaus 1994) to minimize resampling of single genets. Shoots were transported to a greenhouse, where they were tagged to identify sites of origin. Each shoot pair was then planted in a 20 cm high plastic pot filled with native sediment and placed in outdoor flow-through estuarine water tanks. To simulate field conditions, tanks contained water 0.6 m in depth and were shaded to 40% ambient light. Approximately 2 days before their pistils emerged (de Cock 1980), maturing inflorescences were covered with 0.25 mm^2 mesh bags to prevent unplanned pollinations.

Eelgrass flowers mature over a 2 to 4 week period (de Cock 1980). During this time, inflorescences were checked for emergent stigmas every 6 to 8 hours. Shoots with emergent stigmas were moved from the large holding tank to a small, flow-free aquarium. Forceps were used to take a single male flower from a pollen donor randomly chosen from one of the three treatments (different population, same population, or same individual). Pollen strands were separated until they floated at the water's surface, and strands were draped across 3 receptive stigmas per inflorescence for 10 minutes (as in Ruckelshaus 1994), enough time for a pollen tube to begin growing (Rhode, personal observation). The plant containing the manipulated female flower was then returned to the larger holding tank.

When stigmata senesced and were no longer receptive (1-3 days after pollination), inflorescences were unbagged and monitored for seed development. Ten days after fertilization, numbers of viable seeds per shoot were counted; viability was scored using

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external characteristics (Harrison 1991). A total of 50 individuals per cross type were used as maternal parents for the outcrossing (maternal and paternal donors from different populations), inbreeding (maternal and paternal donors from the same population), and self-fertilization treatments. Plants that died before setting seed or whose pre-fertilization history was questionable were not used in the final analysis. Neither pollen donors nor receptive females were used for more than one cross, rendering crosses and all treatments independent. Fitness, calculated as total seeds produced by the experimental cross, was scored for each type of cross. To increase replication, the 1998 experiment was repeated in 1999.

Statistical Analyses

Differences in mating success (binary: seed produced or not) and seed production (number of seeds from 3 potentially-fertilized stigmata) among the three main cross types (outbred, inbred, selfed) were determined with resampling analysis. The three possible comparisons for each response variable were outbred vs. inbred, outbred vs. selfed, and inbred vs. selfed. For each comparison, the data matrix was resampled (with replacement) 1×10^4 times, and the difference between mating type means was calculated for each resampling run. The observed difference between means was then compared to the distribution of resampled values to calculate the probability of obtaining the observed value by chance alone. Because three pairs of means were compared, a p value of 0.05 / 3, or 0.0167, was used as the critical value for statistical significance.

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RESULTS

Non-parametric ANOVA revealed no effects of year by treatment interaction (Mann-Whitney; $p > 0.05$) on the success of crosses or on seed production, so I pooled data from 1998 and 1999. Like Ruckelshaus (1995), this study found a significant effect of maternal source on % successful crosses (Kruskal-Wallis; $n = 25$, $p = 0.0327$). Values for both fertilization success and seed production were lowest in maternal plants from Broad Bay, regardless of mating treatment. Pollen source did not affect the success of fertilization (Kruskal-Wallis; $n = 25$, $p = 0.7604$) or the number of seeds produced (Kruskal-Wallis; $n = 25$, $p = 0.4849$). In the analyses, data were pooled for all types of outbred, inbred, and selfed crosses, regardless of the maternal/paternal combination. Treatment effects remained significant whether or not crosses with Broad Bay mothers were excluded.

Mating type significantly affected mating success (Figure 3). Resampling analysis ($n = 1 \times 10^4$) showed that success (ability to produce any seed) of selfed crosses was greater than that of outbred crosses ($p = 0.0130$); no other paired comparisons were statistically significant at the critical $p = 0.0167$ (selfed vs. inbred: $p = 0.042$; inbred vs. outbred: $p = 0.644$; Figure 3). Mating type also had a significant effect on number of seeds produced per cross (Figure 4). Selfed matings produced more viable seeds per mating than either inbred ($p = 0.0137$) or outbred ($p = 0.0004$) crosses. There was no difference in number of seeds produced by inbred vs. outbred crosses ($p = 0.236$).

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DISCUSSION

In this greenhouse experiment, 37% of all crosses produced seeds. This was lower than estimated rates of success in natural field populations (72%; Churchill and Reiner 1978) and in Ruckelshaus' 1995 laboratory experiment (67%). The reasons for this discrepancy are unclear. Outbred, inbred, and selfed matings all produced seeds in this experiment. Neither cross success nor seed production showed any evidence of the inbreeding depression demonstrated in other eelgrass populations (Ruckelshaus 1994, Reusch 2001). Instead, outbreeding depression was evident in the reduced success of these matings relative to selfed matings. Seed set from inbred and outbred matings was comparable, possibly because genetic diversity is partitioned approximately equally at the within- and among-bed levels in these populations (Rhode 2002, Chapter 2). Thus, results of these breeding experiments indicate that there is no intrinsic post-zygotic barrier to outcrossed, inbred, or selfed matings in eelgrass from these three morphologically and genetically diverse Chesapeake Bay eelgrass beds. However, protogyny and interbed distance, both potential pollination barriers in natural populations, were overcome with the design of this greenhouse experiment. In nature, both selfed (within a single inflorescence) and outbred crosses might occur infrequently.

Matings using maternal plants from Broad Bay had less success and produced fewer seeds than those using maternal plants from Brown's Bay or Allen's Island. In a separate chapter (Rhode 2002, Chapter 4), transplants taken from Broad Bay also produced less biomass than plants from Allen's Island, less total blade area than plants

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from Brown's Bay, and fewer seeds per reproductive shoot than plants from Brown's Bay (Rhode 2002, Chapter 4). A survey of eelgrass reproductive output (Harwell and Rhode, *in prep*) revealed that regional (km scale) variation in reproductive investment is stronger than local (m scale) effects. Rhode (2002, Chapters 2 and 4) noted significant differences in morphology, shoot density, and reproductive output among these beds, and reproductive output in this study seems to mirror vegetative success.

In contrast to these results, studies in Washington, USA and the Baltic Sea showed no evidence of self-fertilization capabilities in eelgrass (Ruckelshaus 1994, Reusch 2000). The ability for self-fertilization in Chesapeake Bay *Zostera marina* might be predicted based on its seemingly biennial life history (Harwell 2000). Since plants do not achieve sexual maturity until their second year, numbers of mature individuals might fluctuate widely from year to year, so self-fertilization ability would ensure seed production. Populations studied by Ruckelshaus (1995) demonstrated home site advantage (less mortality, faster growth) and would be expected to maintain high fitness levels by inbreeding or local mating. Instead, most seeds produced in the field and greenhouse were products of outbred matings. These data support Ruckelshaus' genetic survey (1998), which revealed high levels of genetic substructuring within and among populations.

Transplant experiments in Chesapeake Bay populations also showed little evidence of local adaptation in these eelgrass populations (Rhode 2002, Chapter 4), suggesting no clear advantage to inbreeding. Contrary to expectation, outbred matings

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produced significantly fewer seeds than self-fertilization. Although self-fertilization was the most successful mating strategy in this greenhouse study, allozyme data show high levels of genetic diversity within beds (Rhode 2002, Chapter 2). There are several possible explanations for this paradox. First, allozymes might not actually be neutral markers. Second, eelgrass beds could be composed of multiple patches of self-fertilizing clones. Third, the strategy that produced the most seeds in this greenhouse study might not be the most common one in natural populations. Fourth, there could be low rates of seed germination or seedling establishment in parent beds. Instead, seeds could serve primarily as dispersal agents, traveling from the parent bed via rafting reproductive shoots (Harwell 2000). Alternatively,. Each mechanism would effectively minimize the contribution of small-scale genetic homogeneity introduced by seeds produced via selfed matings.

Environmental structure and demography can constrain populations from panmixia or their most adaptive mating systems (Shields 1993, Waser 1993b). Aspects of the physicochemical or biotic environment might constrain dispersal patterns, impacting mating systems and population fitness. Extinction or founder events can fragment populations spatially, and microhabitat-induced differences in flowering times (e.g. Stanton and Galen 1997) can divide populations temporally, potentially curtailing long-distance gamete exchange. Protogyny could also reduce the incidence of self-fertilization.

One caveat to this work is that fitness measurements were made at only a single

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point in the organism's life history (seed set), and the relative contributions to fitness of seedling production and adult shoot growth are unknown. Phillips *et al.* (1983) predicted that seeds were the most important life history stage during which selection on eelgrass could act, and Ruckelshaus (1994, 1995) found that inbreeding depression was usually expressed as differential germination success rather than seed set. Thus, it is possible that inbreeding depression occurs at later life stages in Chesapeake Bay eelgrass. Although this was not tested explicitly, reciprocal transplant experiments (Rhode 2002, Chapter 4, Figure 12) showed evidence of interpopulation variation in germination success of seeds produced *in situ*.

Ruckelshaus (1994) also demonstrated outbreeding depression in eelgrass seedlings seven months post-fertilization. Longitudinal studies of seed fate would strengthen the experiments reported here and test more rigorously for outbreeding depression. Genetic surveys reveal that eelgrass beds in many areas are mosaics consisting of interspersed clones (Reusch *et al.* 1999a, Reusch *et al.* 1999b). This structure is due, in part, to vegetative reproduction and re-establishment of bare patches by recruited seeds, but it could be reinforced by *in situ* self-fertilization.

A review by Waser (1993b) concluded that inbreeding depression was more common than outbreeding depression in plants. Nevertheless, in spite of the potential for negative fitness consequences (i.e., reduced heterozygosity), some populations engage in exclusive inbreeding or selfing. This can be a mechanism to increase fitness through preservation of locally adapted gene complexes; it can also be coincident with

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demographic fragmentation. Over evolutionary time, plants have developed many pre- and post-zygotic mechanisms to ensure specific types of mating systems. To prevent self-fertilization, which usually produces low-fitness offspring, many bisexual plants such as eelgrass have evolved features like flowering asynchronicity; these might also be evolutionary remnants with little current adaptive value. Data in this study show that, in spite of at least some flowering asynchronicity, eelgrass can produce successful seeds via inbreeding.

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Figure Legends

Figure 1. Morphology of eelgrass reproductive shoot. On a single inflorescence, the approximate ratio of ♀ to ♂ flowers is 1 : 2.

Figure 2. Map of Chesapeake Bay (Virginia, USA). Sites from which plants for artificial crosses were taken are labeled.

Figure 3. Mean (+/- 1 standard error) proportion of crosses that produced viable seeds in outbred, inbred, and selfed matings. Letters beneath bars indicate pollen and ovule donors for each cross. A = Allen's Island, B = Brown's Bay, and BR = Broad Bay. Overall n = 35 to 44 per cross type. Number above bar is actual n per specific cross, which ranged from 5 to 21.

Figure 4. Mean (+/- 1 standard error) number seeds (of 3 possible) produced per cross in outbred, inbred, and selfed matings. Symbols as in Figure 3.

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Figure 1

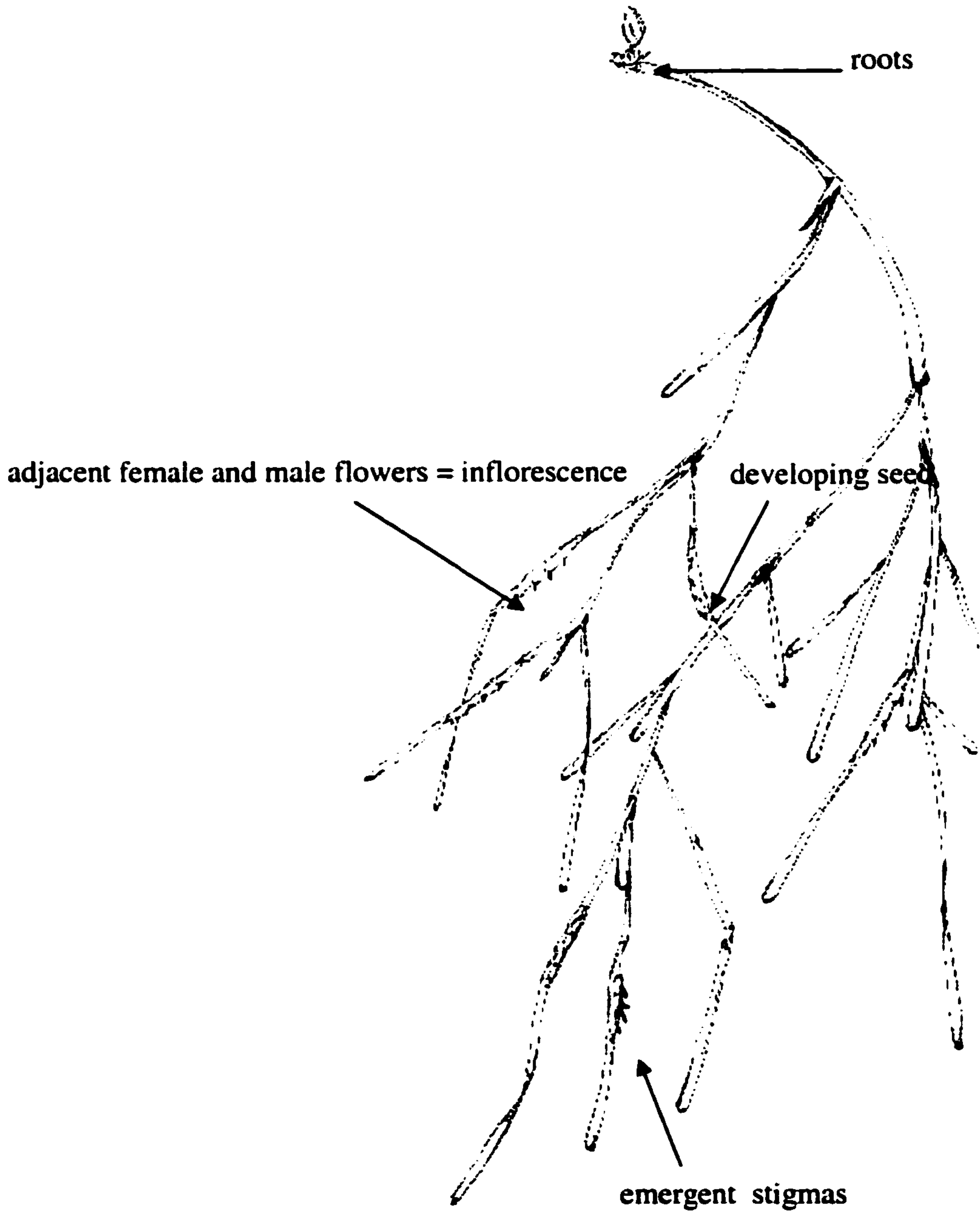

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Figure 2

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Figure 3

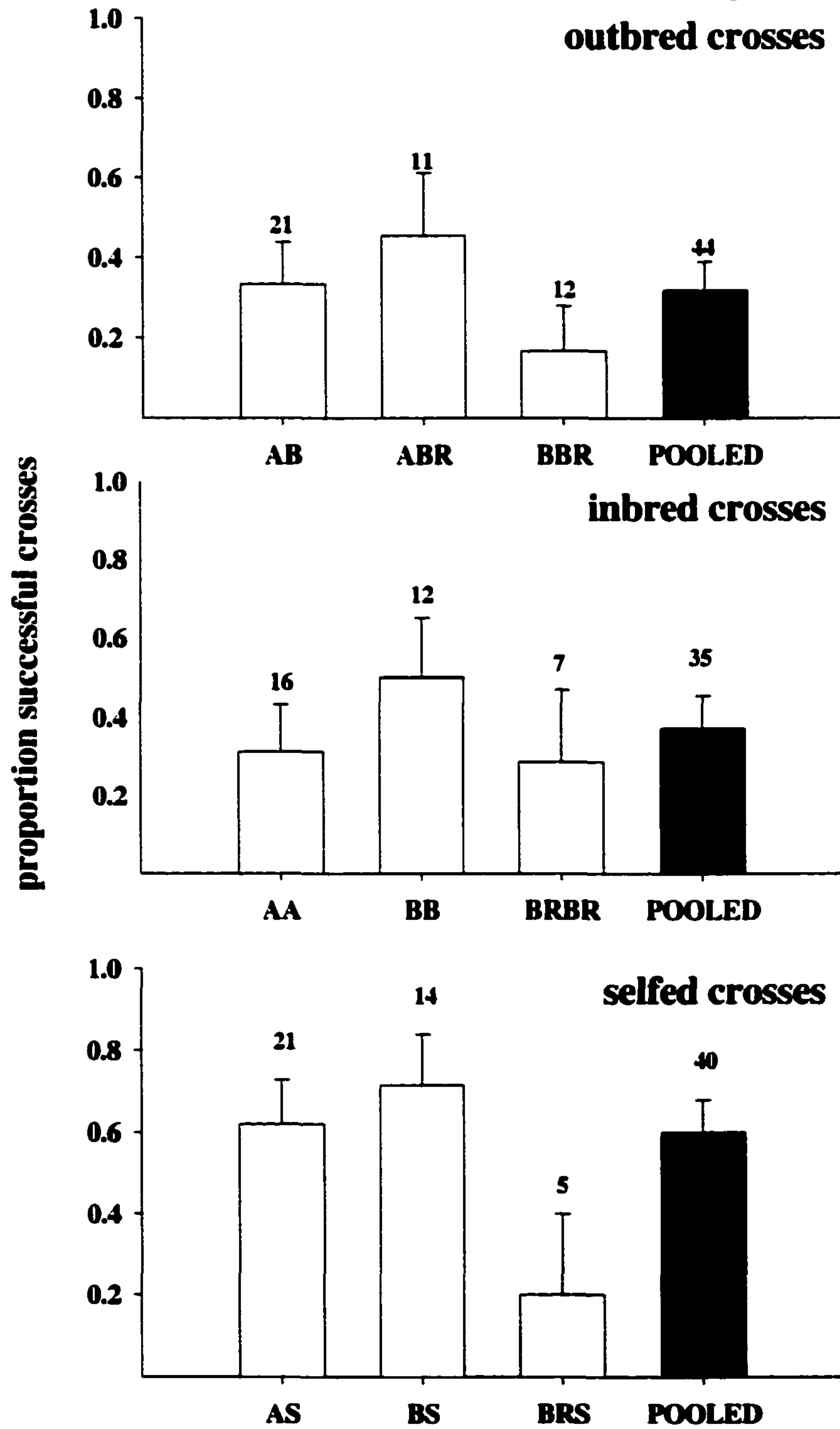

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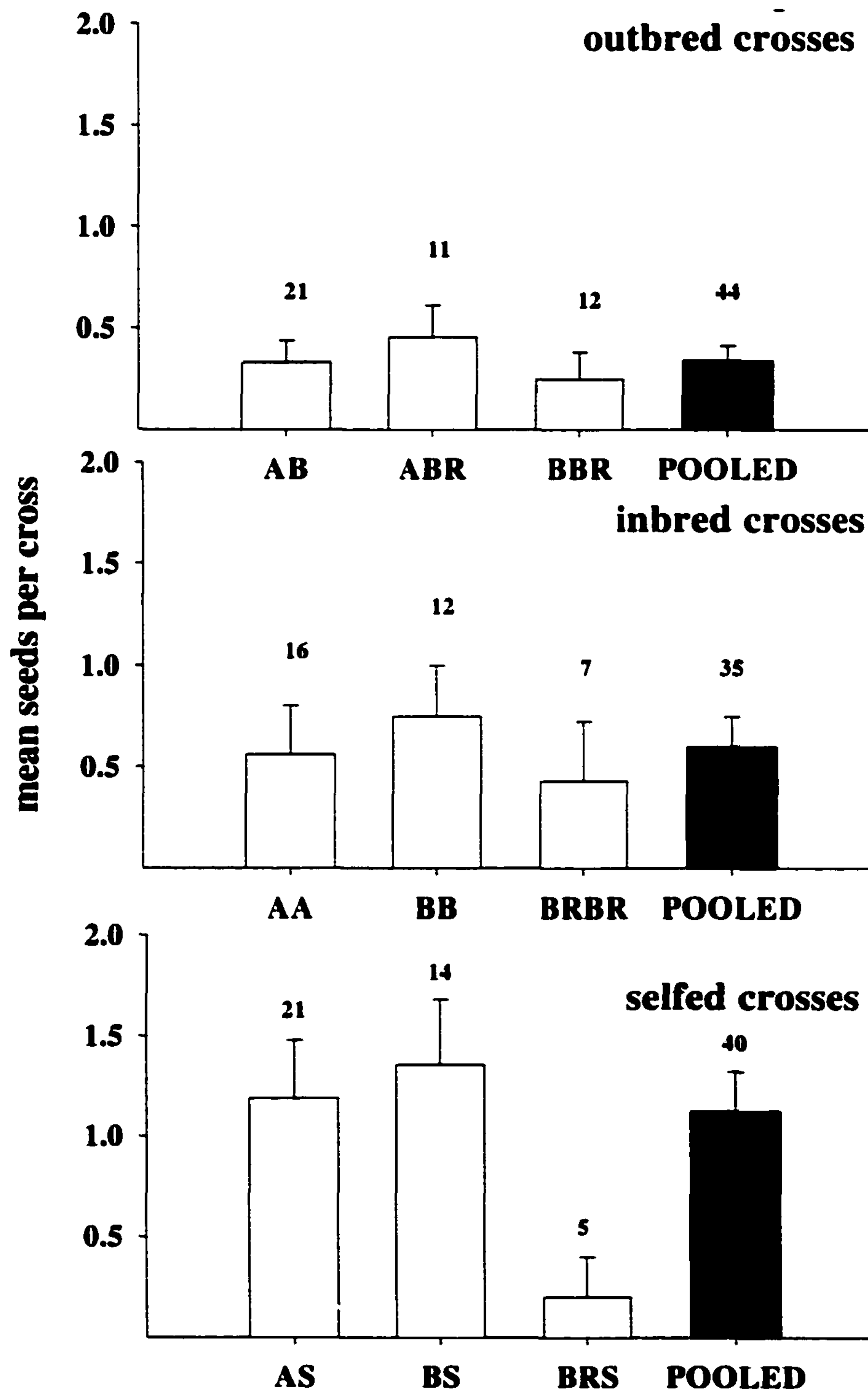
Figure 4



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Chapter 4: Tests for Local Adaptation in Chesapeake Bay (Virginia, USA) Populations of Eelgrass, *Zostera marina* L

ABSTRACT

Eelgrass (*Zostera marina* L.) is the Northern hemisphere's dominant marine angiosperm. Beds of eelgrass exhibit considerable morphological and genetic differences on both geographic and local scales. These could be due to phenotypic plasticity, isolation by distance, or local adaptation. Reciprocal transplants of mature plants and seeds within four phenotypically, genetically, and spatially different Chesapeake Bay eelgrass beds were used to distinguish among these alternatives. Vegetative (blade morphology and density) and sexual (seed production) performance of these transplants were monitored periodically for one or two years to test whether eelgrass fitness was affected by transplant site, source from which plants were taken, or the interaction between site and source. In the first experiment, which included two sites, both site and source significantly influenced vegetative and sexual fitness. Site x source interactions, potentially signifying local adaptation, were less commonly significant. Thus, evidence for local adaptation was equivocal in Experiment I. In the second experiment, which included four sites, most variation in vegetative and sexual fitness could be attributed to site effects only, and there was no clear evidence of local adaptation. However, shoot density and number of seeds per reproductive shoot were higher at native sites, providing some evidence of being locally adapted. Differences in vegetative fitness of transplanted

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seedlings were due entirely to site effects; there was no consistent effect of seed biomass or other maternal or source influence. Since source effects were common and long-lasting in transplants of adult shoots but absent in seed transplants, developmental canalization within these beds was hypothesized. As evidence for home-site advantage was uncommon and inconsistent among years and traits, local genetic adaptation seems relatively unimportant for Chesapeake Bay eelgrass beds. Instead, phenotypic plasticity appears to be of primary importance in maintaining plant performance in these temporally and spatially heterogeneous estuarine environments. These studies thus suggest that current restoration protocols, which choose large, robust beds as sources for transplant material, provide a reasonable management strategy for Chesapeake Bay eelgrass beds. Optimal management strategies may differ elsewhere, as population biology of eelgrass is known to vary considerably across its wide geographic range.

keywords: canalization, environmental effects, local adaptation, phenotypic plasticity, reciprocal transplants, *Zostera marina*

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INTRODUCTION

Populations can adapt to spatially variable environments through phenotypic plasticity, local adaptation or a combination of these. Phenotypic plasticity, the ability of an organism to alter its morphology or biochemistry in response to local conditions, can enhance a genotype's ability to colonize novel habitats or react to shifting environmental conditions. It can also preclude local adaptation (Via and Lande 1985, Sultan 2000), especially if developing or maintaining plasticity incurs little fitness cost. The degree of local adaptation can be used to predict the success of restoration projects, risk of contaminating remnant populations with maladaptive genotypes, and susceptibility to extirpation.

Models predict that fitness costs drive plasticity to evolve more frequently than specialization (Van Tienderen 1997). Experimental manipulations have shown that phenotypic plasticity for morphological and sexual traits is common in plants, whose environments are highly variable in both space and time, often precluding effective genetic adaptation to these shifts (Sultan 2000). Phenotypic plasticity is also common in sessile marine animals. This presumably results from many of the same constraints of immobility as in plants. Plasticity may also be a consequence of low genetic diversity (Barrett *et al.* 1993) or because the progeny of many pelagically-dispersed species are distributed in unpredictable patterns into highly variable environments (Warner 1997). Plasticity is favored when individuals can control neither their environment nor the placement of themselves or their offspring within that environment.

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Local adaptation occurs when selection favors different genotypes under different environmental conditions. The degree of local adaptation depends on the intensity, direction, and spatial variance in selection, the mode of inheritance, mobility of individuals and gametes, and amount of genetic variation within the meta-population (Linhart and Grant 1996). Ultimately, local adaptation requires that populations experience dissimilar environmental regimes and have genetic variation upon which selection can act. Populations with strong genetic structure are often locally adapted. Conversely, high levels of gene flow can destroy locally adapted traits (Sork *et al.* 1998) unless selection acts on each new generation. Reciprocal transplants, which move organisms from native to foreign habitats and pair different phenotypes in common sites, are one of the best means of differentiating between phenotypic plasticity and local adaptation (Schemske 1984, Antonovics *et al.* 1988, Schmitt and Gamble 1990), with enhanced performance of an individual in its home territory providing strong evidence for local adaptation.

Rigorous empirical tests of local adaptation have found numerous examples in nature (Mopper and Strauss 1998). Montalvo and Ellstrand (2000) found an inverse relationship between genetic distance and fitness in *Lotus scoparius* transplants and concluded that these shrubs had increased performance at home sites. Locally adapted traits have been observed between plant populations separated by distances as short as a few centimeters (Linhart and Grant 1996), but local adaptation over short distances or between sites with moderately different environmental conditions is rare (Rice and Mack

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1991, Galloway and Fenster 2000). Local adaptation is most common along strong environmental gradients (Linhart and Grant 1996), in coadapted species, such as parasites and their hosts (Kaltz and Shykoff 1998, Lively 1999), and in organisms with sessile adult stages (Linhart and Grant 1996), especially plants (Heslop-Harrison 1964).

Eelgrass (*Zostera marina* L) is a benthic marine plant with a high intrinsic level of phenotypic plasticity (Backman 1991). High phenotypic plasticity might be expected in eelgrass because it is sessile, populations experience annual cycles of environmental extremes, and propagules can disperse potentially long distances (Harwell and Orth 2002) via floating reproductive shoots (although genetic evidence indicates that successful establishment is apparently rare (Rhode 2002; Chapter 2)). Conversely, local adaptation is favored over phenotypic plasticity when there are marked and temporally stable genetic and environmental differences among populations. These conditions are met by many eelgrass populations.

Z. marina populations extend throughout the temperate zone of the Northern hemisphere. Eelgrass shows much morphological (van Lent and Verschuure 1995, Backman 1991, Phillips and Lewis 1983) and genetic (Reusch 2001) variation over this geographic range. Even within Chesapeake Bay, eelgrass lives under diverse environmental conditions, and thus eelgrass beds provide an interesting natural system in which to conduct studies of phenotypic plasticity and local adaptation. *Z. marina* growth and fitness are affected by many factors, among the most important of which are light and temperature (Wetzel and Neckles 1986). Moreover, an allozyme study by Williams and

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Orth (1998) indicated that genetic diversity of Chesapeake Bay eelgrass is strongly partitioned among subpopulations, with an average F_{ST} value of 0.335 among natural beds. Population substructuring (F_{IS} and F_{ST} ; Wright 1978) is greater for Chesapeake Bay eelgrass than for many other plant species (Wendel and Parks 1985, McCauley 1998, Lin 2001), including most seagrasses (Reusch 2001). These high F_{ST} values could be attributed to limited dispersal, which also promotes local adaptation. Data from floating traps indicate that most of eelgrass' hydrophilous pollen travels less than 3 m from its source flower (Ruckelshaus 1996), and uprooted mature shoots rarely become re-established (Ewanchuk and Williams 1996). Seeds themselves are negatively buoyant and move little once released from their parent shoot (Orth *et al.* 1994). These demographic data make it likely that beds will be strongly genetically distinct, as gene flow among beds might be limited.

The majority of gene flow in Chesapeake Bay eelgrass seems to occur through movement of seed-bearing shoots, which break off from their vegetative neighbors and can float considerable distances (Orth *et al.* 1994, Patterson *et al.* 2001, Harwell and Orth 2002). Such floating shoots have been collected up to 35 km from their nearest likely source (Harwell and Orth 2002). While seed dispersal surveys suggest that eelgrass populations could experience substantial gene flow (Harwell and Orth 2002), genetic differences among Chesapeake Bay eelgrass populations are marked (Williams and Orth 1998; Rhode 2002, Chapter 2). Since eelgrass life stages other than seed-bearing reproductive shoots have very low dispersal capabilities (Orth *et al.* 1994, Ewanchuk and

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Williams 1996, Harwell 2000), successful movement and establishment of novel genotypes is probably infrequent, contributing to the observed genetic subdivision. Selection could also create this genetic structure, and the structure could be reinforced by limited dispersal. Local adaptation could also be promoted by *Zostera marina*'s life history, which includes substantial clonal reproduction and inbreeding (Rhode 2002, Chapter 2). Olesen (1999) concluded that vegetative production was more important to eelgrass persistence than sexual reproduction. Flowering shoot production in the Danish sites on which that study focused was an order of magnitude less than in Chesapeake Bay, and rates of vegetative growth are probably also higher for Chesapeake Bay eelgrass.

Early research noted consistent variation in *Zostera marina* shoot morphology among beds (Bak 1980); some differences in shoot length and width were associated with sediment characteristics (Short 1983) or current regime (Ruckelshaus 1994). When transplanted to a diverse array of habitats, eelgrass quickly changed morphology; this was attributed to the species' exceptional amount of phenotypic plasticity (Phillips and Lewis 1983, Dennison et al 1987), although a portion of the variance could be attributed to genetics (Backman 1991) or canalization. Short-duration common-garden experiments revealed links between environmental factors and fitness proxies such as survival, growth rate, blade length and width, flowering density, seed germination rates, and reproductive strategy (semi-annual vs. perennial) (Orth 1977, Phillips and Lewis 1983, Dennison and Alberte 1986, Backman 1991, Ruckelshaus 1994, van Lent and Verschuure 1995, Phillips

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1996, van Katwijk *et al.* 1998). Nearly all of the aforementioned studies showed an interaction between genotypic and environmental effects, and this interaction might be attributed to local adaptation. It is important to note that, for all traits studied, there was a genetic (or source) limit to the amount of plasticity plants could express.

Several questions about local adaptation in eelgrass remain unexplored. First, no study has monitored both performance (growth) and fitness (seed production) of reciprocal transplants over time scales > 1 year. Thus, the potential role of trait canalization in producing the observed native advantage remains a possible alternative explanation to genetic control. The persistence of native advantage is unknown, and the relationship between plant survival, growth, and seed production is unexplored. The spatial scales over which local adaptation occurs also have not been examined, nor has local adaptation among more than two populations been tested rigorously. The current study addresses all of these issues.

This work's purpose was to determine whether phenotypic variation among Chesapeake Bay eelgrass is best explained by phenotypic plasticity, non-selective genetic structure, or differential adaptation to diverse local environmental conditions. Reciprocal transplants of shoots and seeds from spatially, ecologically, and genetically different areas were used to test this. For the beds of interest, measures of vegetative vigor and seed output were combined to make accurate estimates of population fitness. Morphology, vegetative performance, and sexual fitness of these transplants was monitored periodically for one (Transplant I) or two (Transplant II) years. In the second

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experiment, germination success of seeds was monitored four months after transplantation, and vegetative fitness of seedlings was checked three months later.

METHODS

Study Organism

In Chesapeake Bay (Virginia, USA), *Zostera marina* occupies a large geographic and ecological range, exhibiting a wide array of morphologies over small spatial and temporal scales (Orth and Moore 1986). Eelgrass occurs in a variety of salinity, temperature, sediment, light, and disturbance conditions, and populations are often exposed to drastic environmental fluctuations in a genet's lifetime. At a single location, salinity can vary as much as 8 psu annually, while water temperature ranges from 0 to greater than 30°C (Wetzel and Penhale 1983). Sediments, light, and temperature also differ dramatically among beds. As a result, eelgrass populations might experience differential selection as a result of these environmental extremes.

Eelgrass performance is affected by many abiotic and biotic factors, including current regime (Fonseca and Kenworthy 1987), nutrient availability (Orth 1977, Moore and Wetzel 2000), light levels (Moore and Wetzel 2000), grazer activity (Jernakoff *et al.* 1996, Duffy *et al.* 2001), and genetics (Reusch *et al.* 1999). Flowering and seed production are controlled by shoot age (Setchell 1929), shoot density (Oleson 1999) and water temperature (Orth and Moore 1986); other factors might also influence sexual reproduction.

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On average, Chesapeake eelgrass seeds germinate in October or November (Moore et al. 1993), and standing shoot stocks remain low until March. Shoot density begins to increase in March and peaks in June and July, with temperatures from 20 – 25°C promoting the most vigorous vegetative growth (Marsh *et al.* 1987). Between April and June, reproductive shoots are found in abundance; individuals from perennial eelgrass populations, like those in Chesapeake Bay, might not produce flowers and seeds until their second year (Setchell 1929, Harwell 2000) or after they have reached a critical size (Ewanchuk 1995). Pollination is most effective when water temperatures are between 14 and 16°C (Silberhorn *et al.* 1983). After fertilization, eelgrass seeds mature on their parent plant and are released in late spring. From July until September, the combination of stressful temperatures >25°C and low light from sediment loading and phytoplankton blooms causes mature plants to defoliate (Moore in Batiuk *et al.* 1992). Rhizomes from the previous spring, newer blades, and seeds revegetate beds beginning in mid-September (Orth and Moore 1983). *Zostera marina* in the Chesapeake Bay has a biphasic cycle of vegetative growth, with growth maxima in midsummer and late fall (Moore in Batiuk *et al.* 1992). Because there is much interannual variation in vegetative and sexual characteristics of this species, it seemed important to monitor populations over seasonal cycles for more than one year.

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Site Selection

Four spatially, morphologically (Rhode 2002, this chapter), and genetically (Williams and Orth 1998) different eelgrass beds were chosen to use in tests of local adaptation: Allen's Island, Brown's Bay, Broad Bay, and Milford Haven (Figure 1). Beds that were chosen had all persisted for more than 65 years, as evidenced by aerial photographs and written records; this avoided gross age differences, which could affect the development of local adaptation and confound our analyses. Only Allen's Island had any history of transplant-related supplementation (from Guinea Marshes, near Brown's Bay) (R. J. Orth, pers. comm.). Eelgrass taken from Allen's Island for these experiments was not collected near any of the 1979 – 1980 transplant sites.

Brown's Bay is a shallow (average depth < 1 m at mean low water) site with eelgrass whose short, wide blades are morphologically distinct from those of plants in adjacent beds (see *Results*). Brown's Bay and Allen's Island beds each cover over 150 ha of bottom (Orth *et al.* 1999), and these beds are separated by only 15 km. The Allen's Island bed, which has a shallow, flat, inshore region sloping to deeper water, has been the source for nearly all Chesapeake Bay eelgrass transplants to date. The Broad Bay bed, near the mouth of Chesapeake Bay, is isolated (Harwell and Orth, in press) and genetically distant (Williams and Orth 1998) from the other three beds. This bed covers less than 2 ha of bottom, and it is composed of numerous discrete patches of shoots.

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Finally, Milford Haven was chosen; this bed, which covers less than 1 ha of bottom area, is geographically and genetically (Williams and Orth 1998) distant from the other beds.

To determine morphological differences among the four eelgrass beds, 100 shoots were collected from GPS-generated random points within each bed. To establish shoot densities in each bed, all shoots within 100, 100 cm^2 quadrats were counted, and length and width of the longest blade of 3 haphazardly-collected shoots was recorded. One-way ANOVA was used to compare the length and width of blades in the four beds, and Tukey's *post-hoc* tests were used to determine which sites differed significantly (Zar 1998, SAS 1999).

Environmental Parameters

Local adaptation develops only when sites vary in their selective regimes, and selection is driven by environmental differences. To quantify environmental variation among sites, periodic measurements of environmental parameters that might affect seagrass fitness were made. Onset™ HOBO-Temps were used to make hourly temperature measurements during the spring (February – April), when temperature triggers flowering (Phillips et al. 1983) and promotes vegetative growth, and in summer (May – July), when stressful water temperatures can contribute to defoliation (Orth and Moore 1986, Moore *et al.* 1997). Technical difficulties made it impossible to take additional temperature measurements. Because temperature data were not replicated, no statistical analyses were run. Temperature measurements were collapsed into % of total

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time (out of approximately 1400 hours) during which temperatures were: 1) ideal for growth (20 – 25 °C) (Marsh *et al.* 1987), 2) ideal for pollination (14 - 16 °C) (Silberhorn *et al.* 1983), and 3) stressful (25 °C or greater) (Moore *in* Batiuk *et al.* 1992). Next, correlation analyses and Principal Components Analysis (PCA) were used to examine relationships between temperature regime and eelgrass performance (total biomass, seed production).

Percent water in sediments, a proxy for grain size and indicator of physical energy and nutrient levels (Price and Coles 1992, Erftemeijer and Middelburg 1993), and percent organic material in the sediment, a proxy for nutrient availability, were measured three times between December 1999 and May 2000. These sediment factors are predicted to affect eelgrass fitness (Short 1983, 1987), although a previous transplant study found no relationship between transplant growth rates and sediment characteristics (Davis 1999). To characterize sediments, syringes (diameter = 5 cm) were used to take 10 cm vertical cores from three haphazard points at each of the four sites. Samples were taken from outside of the transplant grids, where sediments had not been homogenized during pre-transplant sieving. Sediment wet mass was measured, sediments were dried for 48 hours at 50 °C, and samples were re-weighed to determine their dry mass. Sediments were then combusted for 6 hours at 500 °C to determine their ash mass, and this value was used to calculate ash-free dry mass. Percent water was calculated as ((wet mass – dry mass) / wet mass) x 100. Percent organic was calculated as ((dry mass – ash-free dry mass) / dry mass) x 100. Differences in all environmental factors were tested using 2-way (time,

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site) ANOVA followed by Tukey tests.

Transplant Experiments

In fall 1997 (Transplant I), a series of reciprocal shoot transplants between Allen's Island and Brown's Bay was used to test adult performance and fitness as a function of source bed. Only two sites were used so that transplants could be monitored often (biweekly) and for more than one year (21 months). Allen's Island and Brown's Bay were used in Transplant I since individuals from these beds were most phenotypically distinct. Transplant sites had approximately equal depths (1 m at MLW) to minimize confounding light effects. First, mature shoots (distinguished from seedlings by larger blade and rhizome size) were collected from each source population. Next, each transplant site was cleared of vegetative material, rhizomes, seeds, and rocks by digging up the area and sieving the sediments through 0.25 cm^2 mesh. A rope grid was erected flush with the bottom, and all shoots and seeds were planted within this grid. Unplanted grid squares separated each planted square; these were used to ensure that plants from different sources did not mix with one another and that no recruitment of adult plants occurred. At both sites mature shoots were planted at a natural field density of 720 shoots per m^2 (Orth and Moore 1986) into 20 randomly arrayed 0.25 m^2 plots (10 native, 10 foreign).

In fall 1998 (Transplant II), reciprocal transplants among the Transplant I sites (Allen's Island, Brown's Bay) and two new sites (Broad Bay and Milford Haven) were

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done. Four sites were used to represent a wider range of eelgrass morphologies, genetic diversity, and habitats. Transplanting was done as above, with 10 native and 30 foreign plots per site, but the planting density was 28% of 1997 values (200 shoots per m^2) (Orth *et al.* 1999). This allowed a greater number of new vegetative shoots to be produced *in situ*.

To determine vegetative fitness, biweekly (Transplant I) or monthly (Transplant II) measurements of shoot density were made. In each 0.25 m^2 (25000 cm^2) plot, all shoots within three haphazard 400 cm^2 quadrats were counted, and this was extrapolated to shoots per m^2. Each month five shoots were collected from haphazard points within each replicate plot. In the laboratory, blade length and width were measured, and total blade area (cumulative length x width x 2 sides to blade) and areal coverage (blade area / bottom area) were calculated. For Transplant I, only the longest blade, the one that is the most morphologically distinct and whose measurements have been recorded by convention (Bak 1980, Short 1983), was measured; all blades were measured for Transplant II. To determine sexual fitness, all reproductive shoots and their seeds were collected from each plot in May 1998 (Transplant I) or May 1999 (Transplant II), and density per m^2 was calculated. These density values were direct counts of all seeds rather than extrapolations, since flowering shoots are patchily distributed and extrapolation might under- or over-estimate sexual fitness (Harwell 2000). In May 1998 (Transplant I) and May 1999 (Transplant II), half of all above- and below-ground biomass was harvested from each plot (Transplant I). Ash-free dry mass per area of each

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plot was determined using the methods described above.

Seed Transplants

Traits might be canalized early in a shoot's morphological development, potentially confounding interpretation of local adaptation in transplanted mature plants (Ruckelshaus 1994). Therefore, reciprocal seed transplants were also done. In late May 1999, seed-bearing shoots were collected from the four field sites (Allen's Island, Brown's Bay, Broad Bay, and Milford Haven). Shoots were stored in flow-through tanks until July 1999, when viable seeds were harvested according to the methods of Orth *et al.* (1994) and moved to clean, aerated flow-through tanks. In October 1999, the mass of a subset of seeds from each site was measured to account for maternal differences in resource allocation, and seeds were transplanted. Seeds were transplanted into a grid of 40 randomly-arrayed 0.0625 m^2 plots (10 native, 30 foreign) of pre-sieved sediments at a natural field density of 1000 seeds/m^2 (Harwell 2000). After four months the number of seeds that had germinated was recorded. After seven months, number of seedlings, seedling blade area and areal coverage were recorded (methods as above). All seedlings were then harvested, and their ash-free biomass was determined as above.

Data Analysis

Data for Transplant Experiments I and II were analyzed separately.

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Before beginning statistical analyses, all data were checked for heterogeneity of variance with a Cochran's test, and data were transformed by the natural-log or square-root as necessary. Using site (destination) and source (bed from which plants were collected) as factors, all vegetative data (shoot width, shoot length, blade area) were analyzed with repeated measures analysis of variance (RMANOVA), more powerful than two-way ANOVA for testing site by source interactions (Horton *et al.* 1991). Two-way ANOVAs followed by Tukey tests were done for each response variable at the time of peak vegetative biomass, when differences among sites were most acute. Reproductive and biomass data were analyzed using two-way ANOVA (site, source) with Tukey *post-hoc* tests (Zar 1998, SAS 1999). Only significant site x source interactions in which local plants had better performance than non-natives were considered evidence of local adaptation.

RESULTS

Results of all statistical analyses are summarized in Table 15.

Site and Bed Characteristics

Measurements of blade morphology prior to transplantation revealed differences among beds in blade area and shoot density, both correlates of vegetative fitness (Table 1, Figure 2). Blade area, surface area of the longest blade in a shoot summed across all shoots in a quadrat, was greatest at Broad Bay and lowest at Brown's Bay. Shoot

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density, the total number of shoots per bottom area, was greater at Brown's Bay than at the other three sites. Although our measurements were made during a single month, other studies support the presence of such marked differences among Chesapeake Bay eelgrass beds (Orth and Moore 1986)

Short-term measurements of environmental factors also showed differences among sites. In spring 2000, water temperatures reached their greatest extremes at Broad Bay, where the 25° C stressful temperature (Moore *in* Batiuk *et al.* 1992) was exceeded during 10% of all measurements. This is significant as even short periods of high temperatures can contribute to eelgrass death. Stressful temperatures were never reached at Milford Haven (Table 2). The proportion of ideal growing hours was highest at Allen's Island and Brown's Bay, while the duration of ideal pollination time was greatest at Milford Haven. Onset of temperatures promoting pollen release varied little among sites, implying that asynchronous flowering would not impede interbreeding among beds.

On average, water content was lowest and organic content highest at Broad Bay and Milford Haven (Table 2), so these sediments might have been the most conducive to vegetative growth (Price and Coles 1992, Erftemeijer and Middelburg 1993). This pattern held even when data were analyzed without May 2001 Milford data (which had almost 3 times as much organic content as other sites and months). It should be noted, though, that measurements of organic matter included surface sediments. These surficial sediments might have contained significant organic rain from the overlying grass canopy, but it is unlikely that this rain was available for uptake by eelgrass roots.

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All measured abiotic factors varied significantly with both site and time. Analysis with 2-way ANOVA also revealed a site by time interaction, so differences between sites were not consistent over the monitoring period. Correlation and Principal Components Analysis showed no significant relationship between any measured abiotic factor and any measure of eelgrass performance or fitness. However, this could be due to the short time over which environmental parameters were measured, small number of sites that were used, or limited range of variables between sites.

Transplant Experiment I: 21 months

<u>Vegetative Performance</u>

Each transplanted plot survived the winter and was monitored in subsequent months. Blade length of transplanted eelgrass was influenced by site, source, and the interaction between site and source (Table 4, Figure 3); blade width was influenced by site and source (Table 4, Figure 3). Blade length and width at both sites were consistently greater in plants from Allen's Island. These trends persisted for most of the twenty-one month duration of the experiment. Source accounted for 71% of the variance in length and 86% of the variance in width (Table 4), but source effects tended to decrease over the course of the experiment. Indeed, changes in both length and width over time were comparable to or greater than the initial differences between sources (Figure 3).

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Area of longest blade per area bottom was affected by site, source, and the interaction between site and source (Table 5, Figure 4). Of these, source was the most important (72% of variance), with shoots from Allen's Island producing more blade area at both Allen's Island and Brown's Bay. Over time, blade area of shoots from the two sources decreased by more than 50% and converged, coincident with a marked decrease in blade area across all treatments. In fact, one-way ANOVA at different times show that the importance of source decreases from time of planting. While source was a significant factor at the vegetative peak in 1998 (2-way ANOVA; p = 0.0001) and explained 37.1% of the variance in blade area, neither source (2-way ANOVA; p = 0.2873) nor the source by site interaction (2-way ANOVA; p = 0.7030) was a significant factor at the 1999 vegetative peak.

Vegetative shoot density was affected significantly by site and the interaction between site and source (Table 5, Figures 4C and 4D). Source had a nearly significant effect on shoot density (p = 0.0653), with shoots growing more densely at their native sites during much of the first year, but this factor explained only 3% of the variance in the data. At the 1998 peak in vegetative biomass, both site and the site by source interaction affected shoot density significantly, whereas in 1999, only site affected shoot density (1-way ANOVA; 1998: site: p = 0.0001; source: p = 0.0001; 1999: site: p = 0.0001). Site and source had significant effects on total shoot biomass in both 1998 and 1999 (Table 6, Figure 5), with plants from Allen's Island producing more above-ground and total

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biomass than those from Brown's Bay in both years. Biomass was generally greater at Allen's Island in both years, regardless of source (Table 6, Figure 5).

Sexual Reproduction

In the Transplant I experiment, all measures of reproductive output differed significantly between the two sites, with generally weaker source effects. In 1998, site and site by source interactions affected number of seeds per shoot (Table 7, Figure 6A), with higher numbers of seeds per shoot at Allen's Island, particularly for plants from Brown's Bay. In 1999, however, seeds per shoot were lower at Allen's Island regardless of source. Site and source influenced the proportion of reproductive shoots in both years (Table 7, Figure 6B), with site accounting for more than half of the variance in this measurement. A higher proportion of shoots were reproductive at Allen's Island than Brown's Bay, and plants from Allen's Island produced more total reproductive shoots. Finally, both site and source had approximately equal influence on seed density (Table 7, Figures 6C-D) in 1998 and 1999. Total seeds were higher at Brown's Bay in 1998 and at the Allen's Island site in 1999. Shoots taken from Allen's Island (source) usually produced more seeds per area than those from Brown's Bay in both years.

Transplant Experiment II: 11 months

Vegetative Performance

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Each transplanted plot survived the winter and was monitored in subsequent months. In the second transplant experiment, blade length was influenced by transplant site and source, while blade width was influenced by site, source, and the interaction between site and source (Table 8, Figure 7). Partitioning of variance revealed that site was the most important factor, explaining 50% of the variance in length and 45% of the variance in width. Both site and source had significant effects on total blade area over the course of transplant monitoring (Tables 9 and 10, Figure 8) and at the 1999 vegetative peak (2-way ANOVA; site: $p = 0.0001$; source: $p = 0.0002$). Site was the most important of these factors, explaining 49% of the variance. Total blade area was highest at the Allen's Island site and lowest at the Broad Bay site; shoots from Milford Haven (source) generally had the highest total blade area regardless of site over the course of the experiment, including at the time of peak vegetative density.

Densities were influenced by site, source, and site by source interactions (Table 9, Figure 8), and overall densities were highest at Allen's Island and Milford Haven and were lowest at Broad Bay (Table 10). Only site affected biomass (Table 11, Figure 9), with plants at Broad Bay having less above and below-ground biomass than those at other sites.

Sexual Reproduction

Site and source influenced seeds/reproductive shoot for Transplant II (Tables 12 and 13, Figure 10A), and site was the more important of these. On average, plants at

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Allen's Island and Milford Haven produced more seeds per reproductive shoot than plants at the other sites. Site and source influenced the proportion of shoots that were reproductive (Tables 12 and 13, Figure 10B). Total seed production per area, the most direct estimate of sexual fitness, was affected only by site (Tables 12 and 13, Figure 10C). Allen's Island was the site at which the most seeds were produced.

Seed Transplant

Dry mass of individual seeds harvested from the four transplant sites differed significantly (Figure 11) (ANOVA: $p = 0.001$), with site accounting for nearly half of the variance in this measure. Seeds from Allen's Island were the largest, while those from Brown's Bay and Milford Haven were the smallest. Germination rates of transplanted seeds varied from 0 – 35% and were highly dependent on planting site. Site had a significant effect on percent germination, blade area, and biomass of seedlings, but source did not (Table 14, Figures 12A-C). Thus, the significant differences in seed size (Figure 11) did not affect any seedling trait. Germination rates were highest at Allen's Island and lowest at Milford Haven. Seedlings also produced the most blade area and biomass at Allen's Island and the least blade area and biomass at Milford Haven.

Transplant Experiment I vs. Transplant Experiment II

To compare Transplant I and Transplant II data and thus separate site and source effects from interannual effects, we re-analyzed Transplant II data using just Allen's

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Island and Brown's Bay as sites and sources. Comparing only these two sites showed that both site and source affected blade area (2-way ANOVA; site: $p = 0.0001$; source: $p = 0.0001$), with plants at or from Allen's Island producing more area than those at or from Brown's Bay. Unlike the Transplant I experiment, blade area was not affected by site*source interactions. Only site affected total seed production (2-way ANOVA, $p = 0.0010$) and seeds produced per shoot (2-way ANOVA, $p = 0.0001$), with both measures greater at Allen's Island. In Transplant I, number of seeds per shoot was also affected by the source from which plants were taken. Other vegetative and sexual fitness parameters were not affected by any of the measured variables.

DISCUSSION

In this study, seeds/m^2 ranged from 100 to 1500, on the same order of magnitude as values reported by Silberhorn *et al.* (1983) (8127 seeds/m^2). This was far fewer seeds than the 30000 seeds/m^2 recorded by Santamaria-Gallegos *et al.* (2000) in annual eelgrass populations from Baja California even though shoot densities in both sites were similar (200 to 3000/m^2 in this study vs. 664 to 2234/m^2 in Santamaria-Gallegos *et al* .(2000)). The proportion of shoots that became reproductive ranged from 2 to 30% in this study, similar to results obtained from a survey of undisturbed, perennial Chesapeake Bay populations (11 to 19%; Silberhorn *et al.* 1983) and to Dutch populations (1 – 34%; van Lent and Verschuure 1995).

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Every vegetative and sexual fitness parameter measured was affected significantly by planting site, and these strong site effects provide evidence for eelgrass plasticity. Site effects had no relationship with any measured environmental parameters. Therefore, it is difficult to speculate about causal relationships between eelgrass fitness and other site characteristics. To further explore the influence of environment on eelgrass performance, future studies should monitor additional environmental variables, including light, and monitoring should be done at closer intervals over the course of the entire study.

Source had a highly significant influence on many measures of adult sexual and vegetative fitness, including blade length, blade width, blade area, total biomass, and seed production. This suggests that genetics had a strong effect on these measures. In Transplant I, though, the strength of source effects often decreased over the time of monitoring, suggesting that the observed effects were due to canalization rather than genetics. On the other hand, source effects tended to become more pronounced over time in Transplant II (*e.g.* Figure 8). These data illustrate the value of a multi-year approach with frequent performance monitoring of all vegetative and sexual fitness proxies. Using multiple monitoring years is especially crucial for studies of temperate species, which have considerable seasonal and annual variability in environmental conditions and in patterns of vegetative and sexual performance.

There were several intriguing instances of enhanced performance at native sites that might indicate local adaptation. For example, longest blade length was often greater

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at native than foreign sites in Transplant I (Figures 3 A and 3B). Shoot densities in this study were comparable to those reported in the literature (Olesen 1999). Shoot density in Transplant I was clearly higher at native sites (Figures 4C and 4D), and we observed a similar trend for longest blade area (Figures 4A and 4B). Nevertheless, those trends were not borne out in the Transplant II experiment or for most other performance measures. Thus, by most measures, these eelgrass populations rarely performed best in their native environments, and we conclude that there is little evidence for local adaptation in our study system. This study found no consistent evidence that eelgrass is locally adapted over an interbed scale, but adaptation could occur over smaller spatial scales, such as within beds. The only vegetative trait that seemed locally adapted was shoot density (Figures 4C, 4D, 8E-H). Shoot density might well be important in establishing populations or maintaining clone dominance. However, other traits such as shoot size, total biomass, and seed production might be more important measures of individual plant fitness.

There were interesting discrepancies between vegetative and sexual fitness measures in Transplant I and in Transplant II; fewer source effects were observed in the second transplant experiment. Differences between Transplants I and II could have been due to the addition of two new sites (Broad, Milford), but analyzing Transplant II data using only the original sites (Allen's, Brown's) still yielded differences, with lower blade area, shoot density, and biomass in the second transplants. The contrast between the Transplant I and II data sets likely reflects the pronounced interannual variation in water

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and, to a lesser extent, sediment quality in the dynamic waters of Chesapeake Bay estuary. The year in which Transplant I morphologies converged and source effects diminished (1999) was the year in which Transplant II morphologies demonstrated few source effects; the latter might have not been expressed since blade areas and densities were much lower in 1999 than 1998. Environmental conditions in 1998 seemed to be more conducive to eelgrass growth than those in 1999, in that 1998 was a dry year, with low turbidity (high light) and low levels of water column nutrients. These temporal differences were reflected in areal coverage estimates of natural populations in these years (Orth *et al.* 2000, 2001). It should be noted that these high levels of temporal variation in environmental conditions can slow or prevent the development of local adaptation (Rice and Mack 1991, Galloway and Fenster 2000).

There are other explanations for interannual variation in eelgrass performance and fitness. Fine-scale genetic structure might have influenced the outcome of the two transplant experiments. Transplanted grass was not taken from identical spots in the source populations, and perhaps the plants selected from the populations for Transplant II were different enough from the Transplant I plants to affect this study's results. Differences could also be attributable to initial planting densities (Harwell and Orth 1999), which differed by a factor of three between the two experiments. Perhaps the stress of crowding in Transplant I exaggerated source effects that might have otherwise been hidden, or perhaps the fact that a higher proportion of shoots were produced *in situ* during Transplant II made site effects more pronounced. A study by van Katwijk *et al.*

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(1998) found that effects of planting density on eelgrass morphology, while initially present, disappeared after an adjustment period. The planting densities used in those studies were only 10-20% of those used here, though. In eelgrass, increased shoot density is often correlated with increased shoot size, as both are products of good environmental conditions (Worm and Reusch 2000); we could have observed this phenomenon in our transplants.

Seedling performance metrics also did not support the hypothesis of local adaptation. Vegetative fitness of seedlings was influenced only by site, in spite of significant maternal (source) influences on seed biomass. This evidence, combined with the decreasing importance of source effects (measured as change in percent variance) over time in Transplant I, could suggest that traits observed in mature shoot transplants, rather than being locally adapted or attributable to some genetic effect, might be canalized. If so, the source effects and site by source interactions found in the ANOVA could be remnants of a source signature that disappears after a season of defoliation and new blade growth. Alternatively, decline of a source signature could be due to lower overall growth and biomass in the second year of Transplant I, to new growth overwhelming trait canalization, or to site effects being much stronger than source effects. In the latter case, the poorer growth conditions later in the experiment might actually be masking important source-specific performance that is expressed under better conditions, such as those present earlier in the experiment. This study found germination rates of 0-35%, rates that were highly dependent on planting site, while previous studies

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found 10 -15% germination rates for eelgrass seeds planted in the field (Harrison 1993, Orth and Harwell 1999). Strong site effects on the performance of field-germinated seedlings supports Ruckelshaus' (1994) observation that selection is most pronounced in the earliest life stages of eelgrass. This strong site-based selection and concomitant phenotypic plasticity might help to explain marked morphological differences among beds. Our results suggest that selective forces among sites and responses among genotypes might be plastic enough to make local adaptation unnecessary to eelgrass fitness. Any local adaptation that does exist is accompanied by strong phenotypic plasticity.

While source affects many aspects of vegetative performance in Chesapeake Bay eelgrass, site has stronger effects on all measures of sexual fitness, so site choice is crucial in ensuring transplants' long-term success. Perhaps the traits most important to look for in a transplant site are similar to those found at Allen's Island: many days with ideal temperature conditions, few days with stressful temperatures, coarse-grained sediments, and low organic matter. It should be noted, though, that these environmental variables might be only correlated with eelgrass performance and might not actually cause these different responses.

Source effects cannot be ignored completely, however. Canalization of traits in adult eelgrass seems to persist, in some instances, for up to one year after the time of transplanting. If this is the case, restorations should use donor beds with the most vigorously-growing grass. Otherwise, extirpated beds can be replaced using donor

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material from the most convenient source. Source can have short-term effects on eelgrass performance, and the period immediately following transplantation can be crucial in the successful establishment of new beds or mitigation of troubled ones. Source populations would ideally be from a similar environment or have similar genetics, but these data suggest that this is not absolutely necessary since plants eventually converge in most vegetative and sexual characteristics. Genetic contamination or the preservation of remnant genetic stock as insulation against unforeseen environmental challenges (i.e., storm events, eutrophication, global climate change) should be the main concerns. Even if the value of current genetic differences is unknown, this diversity might become important in the species' future as it encounters novel conditions, and lack of genetic diversity could lead to inbreeding depression.

Future studies should attempt to make direct correlations between sexual fitness and environmental parameters, addressing variables such as light quantity and nutrient levels over several full annual cycles. Net reproductive output could be the best integrative measure of fitness in organisms with a sexual component to their life history, but the real question in clonal organisms like eelgrass is the relative importance of vegetative and sexual processes in population formation and retention/maintenance. Knowing the relative importance of these would allow restoration strategies to be tailored to individual population needs. Temporal variation and the logistics of tracking seed dispersal and establishment make estimating the relative contribution of vegetative and

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sexual reproduction to eelgrass fitness difficult at best, but this vital information could be used to identify appropriate source populations.

There are likely to be important tradeoffs between vegetative and sexual fitness (Zhang and Wang 1994) in eelgrass, so one can predict that populations with particularly poor seed output would compensate with vigorous vegetative growth. This was not the case, however. Production of reproductive shoots and seeds were directly related (as in Fukuda and Tsuchiya 1987), and sites and sources with high sexual fitness also had high vegetative output (e.g. Figures 8 and 10). This suggests that there is a threshold vegetative fitness level above which seed production is possible. Indeed, studies of several other plant species reveal concomitant variation in sexual and vegetative output rather than tradeoffs (Eckert *et al.* 2000, Dorken and Eckert 2001). Alternatively, the age structure of populations in this study might differed from one another. Like most perennial eelgrass worldwide, Chesapeake Bay eelgrass does not produce seeds until its second year (Setchell 1929). Perhaps plants from some sources were old enough to reproduce sexually and have exceptional vegetative output, while those from other sources were immature, pre-reproductive seedlings. Finally, it is likely that good sites promote vegetative and sexual fitness, while bad sites compromise both. Any growth costs incurred by reproduction seem to be overwhelmed by site effects in ideal sites, and these costs might be exacerbated by conditions in poor sites.

Organisms that alter their morphology in response to environmental changes can increase their fitness (Caldwell 1987). Phenotypic plasticity could be the factor that

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ensures eelgrass survival, and observed genetic patterns could be the results of neutral demographic processes. Terrestrial plants alter their genet and ramet architecture in response to environmental differences, foraging for resources more efficiently via morphological changes (De Kroon and Hutchings 1995). Patterns of eelgrass morphology within and among Chesapeake Bay beds could be explained by optimal resource foraging. Terrestrial plants also alter allocation to vegetative and sexual fitness in response to their environments (Zhang and Wang 1994). Perhaps this is one explanation for spatial and temporal differences in flowering and seed production.

Plasticity is common in plants (Sultan 2000) and sessile marine organisms (Barrett *et al.* 1993) since they have little ability to move from inhospitable environments and rarely control the environment into which their offspring are dispersed. The plasticity observed here could have evolved in a novel, unpredictable environment, or it could be an evolutionary remnant. Perhaps historic bottlenecks, which decimated eelgrass abundance and might have reduced genetic diversity, eliminated locally adapted populations and left only those that were phenotypically plastic. Phenotypic plasticity could then be sustained if its fitness costs are low even if it confers little evolutionary advantage. Over time, populations might lose genetic diversity unless neutral evolutionary forces retain site-specific genetic structure or some as-yet unmeasured genetic (source) factor promotes fitness.

Preservation of eelgrass habitat is crucial both economically (Costanza *et al.* 1997) and ecologically, so many agencies, including those around Chesapeake Bay, have

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undertaken transplantation projects to restore threatened or extirpated populations. Data from this study allow the formation of practical recommendations for eelgrass restoration ecology in Chesapeake Bay and its tributaries. These findings suggest that seed transplants might be the best way to restore decimated Chesapeake eelgrass beds. Seed transplants seem to avoid canalization and source signatures associated with mature shoot transplants; their low germination rates are offset by the relatively low labor cost associated with their planting (Orth *et al.* 1998). However, if transplant site conditions are particularly poor, which is often the case with eelgrass transplants, retaining a source signature in transplanted shoots might be desirable as it would boost fitness. Chesapeake managers should take all these factors into account as they attempt to restore extirpated populations or subsidize existing ones.

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TABLES

TABLE 1. Results of 1-way ANOVAs testing differences among sites in area of longest blade per shoot and in vegetative shoot density. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
longest blade area:					
site (3)	34004245254.52	11334748418.00	17.38	**0.0001**	10.40
error (396)	292893051395.51	652112861.82			89.60
shoot density:					
site (3)	10583639.40	3527879.80	9.52	**0.0001**	6.71
error (396)	147187532.67	370749.50			93.29

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TABLE 2. Percent measured hours (of approximately 1400 hours) during Spring 1999 with ideal water temperatures for growth and pollination, and stressful water temperatures. Mean (± 1 SE) hours ideal light levels. Mean (± 1 SE) percent water and organic matter in sediment at 3 different times. Measurements were made at Brown's Bay, Broad Bay, Milford Haven, and Allen's Island. See Table 3 for ANOVA results.

	% of hours		
site	ideal growth	ideal pollination	stressful
Brown's	21.73	19.65	5.31
Broad	11.7	16.2	9.30
Milford	0.17	31.15	0
Allen's	21.25	17.61	5.21

	% water, 01/00	% water, 12/00	% water, 05/01
Brown's	37.58 (3.12)	28.77 (0.49)	28.56 (0.79)
Broad	26.11 (2.47)	25.56 (0.10)	26.03 (0.85)
Milford	29.31(0.52)	26.65 (0.77)	20.87 (0.87)
Allen's	29.82 (1.79)	29.03 (1.65)	29.88 (1.31)

	% organic, 01/00	% organic, 12/00	% organic, 05/01
Brown's	1.360 (0.270)	0.508 (0.028)	0.447 (0.060)
Broad	2.081 (1.432)	0.652 (0.113)	0.570 (0.121)
Milford	0.987 (0.030)	0.625 (0.081)	3.752 (0.510)
Allen's	0.849 (0.028)	0.610 (0.038)	0.508 (0.028)

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TABLE 3. Results of 1-way repeated measures ANOVAs (RMANOVAs) testing differences among sites in sediment % water and sediment % organic over 3 measurement times. n = 3 for each time at each site. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	P	% variance explained
% water:					
site (3)	231.22	77.07	11.13	**0.0001**	34.52
time (2)	122.77	61.39	8.86	**0.0013**	18.33
site*time (6)	149.62	24.94	3.60	**0.0109**	22.34
error (24)	166.20	5.53			24.81
% organic:					
site (3)	6.29	2.10	3.48	**0.0315**	14.23
time (2)	4.64	2.32	3.85	**0.0354**	10.50
site*time (6)	18.82	3.14	5.21	**0.0015**	42.58
error (24)	14.45	0.64			32.69

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TABLE 4. Transplant Experiment I. Results of 2-way RMANOVAs testing differences among sites and sources in length and width of longest blade. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
length:					
site (1)	1372.77	1372.77	67.29	**0.0001**	17.66
source (1)	5565.08	5565.08	272.78	**0.0001**	71.59
site*source (1)	305.06	305.06	14.95	**0.0007**	3.92
error (28)	530.44	20.40			6.82
time (16)	31372.73	1335.80	101.91	**0.0001**	77.33
time*site (16)	1140.83	71.30	5.44	**0.0001**	2.82
time*source (16)	2458.86	153.68	11.72	**0.0001**	6.06
time*site*source (16)	145.70	9.11	0.69	0.7999	0.36
error (416)	5452.81	13.09			13.44
width:					
site (3)	1.44	1.44	9.36	**0.0047**	3.06
source (3)	40.51	40.51	263.67	**0.0001**	86.12
site*source (9)	0.63	0.62	4.07	0.0529	1.33
error (114)	4.46	0.15			9.49
time (5)	81.54	5.10	41.64	**0.0001**	52.33
time*site (15)	5.80	0.36	2.96	**0.0001**	3.73
time*source (15)	9.56	0.60	4.88	**0.0001**	6.14
time*site*source (570)	2.10	0.13	1.07	0.3778	1.35
error (570)	56.79	0.12			36.45

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TABLE 5. Transplant Experiment I. Results of 2-way RMANOVAs testing differences among sites and sources in blade area of longest blade and shoot density. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
longest blade area:					
site (1)	23.01	328.12	34.12	**0.0001**	7.62
source (1)	218.52	3951.95	324.09	**0.0001**	72.18
site*source (1)	43.00	160.83	63.77	**0.0001**	14.20
error (27)	18.21	9.66			6.02
time (16)	1048.24	865.15	100.88	**0.0001**	66.04
time*site (16)	43.03	36.74	4.14	**0.0001**	2.71
time*source (16)	174.72	164.90	16.81	**0.0001**	11.01
time*site*source (16)	40.68	5.49	3.91	**0.0002**	2.56
error (432)	280.56	8.02			
shoot density:					
site (1)	4466391.75	4466391.75	38.58	**0.0001**	34.21
source (1)	426053.84	426053.84	3.68	0.0653	3.26
site*source (1)	4922805.13	4922805.13	42.52	**0.0001**	37.70
error (27)	3241617.49	115772.05			24.83
time (16)	209277770.39	12310457.08	106.19	**0.0001**	68.87
time*site (16)	21433255.57	1260779.74	10.88	**0.0001**	7.05
time*source (16)	6746699.69	396864.69	3.42	**0.0001**	2.22
time*site*source (16)	11222447.61	660143.98	5.69	**0.0001**	3.69
error (432)	55183709.51	115932.16			18.16

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TABLE 6. Transplant Experiment I. Results of 2-way ANOVAs testing differences among sites and sources in above-ground, below-ground, and total biomass at vegetative peaks in 1998 and 1999. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
1998					
above-ground biomass					
site (1)	5080.29	5080.29	6.09	**0.0185**	12.57
source (1)	5201.36	5201.36	6.24	**0.0172**	12.87
site*source (1)	121.21	121.21	0.15	0.7052	0.30
error (36)	30016.72	833.80			74.26
below-ground biomass:					
site (1)	133.01	133.01	1.05	0.3133	2.76
source (1)	113.30	113.30	0.90	0.3496	2.35
site*source (1)	40.24	40.24	0.32	0.5757	0.83
error (36)	4540.76	126.13			94.06
total biomass:					
site (1)	10.19	10.19	2.68	0.1105	6.11
source (1)	3.35	3.35	5.14	**0.0295**	11.72
site*source (1)	0.14	0.14	0.02	0.8990	0.04
error (36)	18.28	0.51			82.13
1999					
above-ground biomass:					
site (1)	724.09	724.09	5.27	**0.0277**	11.08
source (1)	815.54	815.54	5.93	**0.0200**	12.48
site*source (1)	45.02	45.02	0.33	0.5707	0.69
error (36)	4950.29	137.51			75.75
below-ground biomass:					
site (1)	0.2301	0.2301	1.58	0.2163	4.03
source (1)	0.2220	0.2220	1.53	0.2244	3.89
site*source (1)	0.0230	0.0230	0.16	0.6928	0.40
error (36)	5.2305	0.1453			91.67
total biomass:					
site (1)	3.9578	3.9578	35.01	**0.0001**	42.30
source (1)	1.2946	1.2946	11.45	**0.0017**	13.84
site*source (1)	0.0346	0.0346	0.31	0.5836	0.37
error (36)	4.0691	0.3362			43.49

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TABLE 7. Transplant Experiment I. Results of 2-way ANOVAs testing differences in measures of sexual fitness in 1998 and 1999. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
1998					
seeds per reproductive shoot:					
site (1)	192.15	192.15	55.08	**0.0001**	57.49
source (1)	1.11	1.11	0.32	0.5758	0.33
site*source (1)	15.39	15.39	4.41	**0.0428**	4.60
error (36)	125.59	3.49			37.57
proportion shoots reproductive:					
site (1)	0.0692	0.0692	79.60	**0.0001**	52.00
source (1)	0.0226	0.0226	25.95	**0.0001**	16.98
site*source (1)	0.0100	0.0100	11.54	**0.0017**	7.51
error (36)	0.0313	0.0009			23.52
seeds/m²:					
site (1)	5364537.05	5364537.05	9.25	**0.0044**	17.02
source (1)	4565961.67	4565961.67	7.87	**0.0080**	14.48
site*source (1)	719371.41	719371.41	1.24	0.2727	2.28
error (36)	20874055.90	579834.89			66.22
1999					
seeds per reproductive shoot:					
site (1)	28.95	28.95	8.70	**0.0060**	19.79
source (1)	6.65	6.65	2.00	0.1674	4.54
site*source (1)	7.56	7.56	2.27	0.1418	5.17
error (31)	103.16	3.33			70.50
proportion shoots reproductive:					
site (1)	0.4255	0.4255	91.78	**0.0001**	63.33
source (1)	0.0622	0.0622	13.41	**0.0008**	9.26
site*source (1)	0.0173	0.0173	3.73	0.0613	2.57
error (31)	0.1669	0.0046			24.84
seeds/m²:					
site (1)	1390086.10	1390086.10	4.80	**0.0361**	11.70
source (1)	1192302.72	1192302.72	4.12	0.0511	10.03
site*source (1)	327323.90	327323.90	1.13	0.2959	2.75
error (31)	8975370.50	289528.08			75.52

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TABLE 8. Transplant Experiment II. Results of 2-way RMANOVAs testing differences among sites and sources in total blade length (sum of lengths of all blades per shoot) and longest blade width. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	P	% variance explained
length:					
site (3)	27109.00	9036.33	79.20	**0.0001**	49.71
source (3)	13658.28	4552.76	39.91	**0.0001**	25.04
site*source (9)	765.20	85.02	0.75	0.6668	1.40
error (114)	13006.12	114.09			23.85
time (5)	80707.08	13451.18	132.51	**0.0001**	45.74
time*site (15)	15126.15	840.34	8.28	**0.0001**	8.57
time*source (15)	5473.53	304.09	3.00	**0.0001**	3.10
time*site*source (570)	5702.45	105.60	1.04	0.3992	3.23
error (570)	69434.13	101.51			39.35
width:					
site (3)	14.34	4.78	42.13	**0.0001**	45.01
source (3)	2.08	0.69	6.10	**0.0007**	6.52
site*source (9)	2.51	0.28	2.46	**0.0135**	7.88
error (114)	12.94	0.11			40.60
time (6)	261.78	43.63	356.71	**0.0001**	68.15
time*site (18)	25.89	1.44	11.76	**0.0001**	6.74
time*source (18)	4.96	0.28	2.25	**0.0022**	1.29
time*site*source (684)	7.82	0.14	1.18	0.1787	2.04
error (684)	83.66	0.12			21.78

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TABLE 9. Transplant Experiment II. Results of 2-way RMANOVAs testing differences among sites and sources in total blade area and shoot density. P values ≤ 0.05 are in bold.

Source (DF)	SS	MS	F	P	% variance explained
total blade area:					
site (3)	41.74	13.91	51.17	**0.0001**	48.81
source (3)	8.92	2.97	10.94	**0.0001**	10.43
site*source (9)	3.86	0.43	1.58	0.1306	4.51
error (109)	30.99	0.27			36.25
time (6)	152.484	38.12	159.16	**0.0001**	52.06
time*site (18)	15.14	1.26	5.27	**0.0001**	5.17
time*source (18)	5.48	0.46	1.91	**0.0317**	1.87
time*site*source (54)	10.56	0.29	1.21	0.1787	3.61
error (654)	109.22	0.24			37.29
shoot density:					
site (3)	4744536.79	1581512.26	110.63	**0.0001**	65.03
source (3)	207371.50	69123.83	4.84	**0.0031**	2.84
site*source (9)	385677.33	42853.04	3.00	**0.0027**	5.29
error (109)	1958494.10	14295.58			26.84
time (6)	19782570.71	2826081.53	216.61	**0.0001**	52.98
time*site (18)	3365644.51	160268.79	12.28	**0.0001**	9.01
time*source (18)	459221.53	21867.69	1.68	**0.0289**	1.23
time*site*source (54)	1215870.86	19299.54	1.48	**0.0105**	3.26
error (654)	12511937.24	13046.86			33.51

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TABLE 10. Transplant Experiment II. Results of Tukey tests following significant ($p \leq 0.05$) 2-way ANOVA testing differences among sites and sources in total blade area.

site	Tukey Grouping	source	Tukey Grouping
total blade area:		*total blade area:*	
Brown's	B, C	Brown's	B
Broad	C	Broad	A
Milford	A	Milford	A
Allen's	A	Allen's	A

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TABLE 11. Transplant Experiment II. Results of 2-way ANOVAs testing differences among sites and sources in above-ground, below-ground, and total biomass (1999). P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	P	% variance explained
above-ground biomass:					
site (3)	495765.54	165255.18	16.92	**0.0001**	28.78
source (3)	59488.35	19829.45	2.03	0.1136	3.45
site*source (9)	44320.45	4924.49	0.50	0.8689	2.57
error (115)	1123274.93	9767.61			65.20
below-ground biomass:					
site (3)	2224.50	741.50	9.60	**0.0001**	17.59
source (3)	447.73	149.24	1.93	0.1280	3.54
site*source (9)	549.41	61.05	0.79	0.6259	4.34
error (115)	9425.14	77.26			74.53
total biomass:					
site (3)	198061.20	66020.40	15.81	**0.0001**	27.94
source (3)	20540.54	6846.85	1.64	0.1843	2.90
site*source (9)	26663.61	2962.62	0.71	0.6992	3.76
error (111)	463551.98	4176.14			65.40

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TABLE 12. Transplant Experiment II. Results of 2-way ANOVAs testing differences among sites and sources in seeds / reproductive shoot, proportion seeds reproductive, and seeds / m^2 (1999). P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
seeds per reproductive shoot:					
site (3)	1285.92	428.64	27.67	**0.0001**	35.17
source (3)	150.42	50.14	3.24	**0.0244**	4.11
site*source (9)	175.05	19.45	1.26	0.2673	4.79
error (132)	2045.17	15.49			55.93
proportion shoots reproductive:					
site (3)	0.7604	0.2535	6.25	**0.0005**	10.05
source (3)	0.7133	0.2378	5.86	**0.0009**	9.43
site*source (9)	0.6143	0.0683	1.68	0.0990	8.11
error (132)	5.4777	0.0406			72.40
seeds/m²:					
site (3)	6927379.17	2309126.39	15.56	**0.0001**	23.66
source (3)	2309126.39	249390.33	1.68	0.1744	7.89
site*source (9)	748171.00	55270.59	0.37	0.9464	2.56
error (132)	19294131.63				65.90

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TABLE 13. Transplant Experiment II. Results of Tukey tests following significant ($p \leq 0.05$) 2-way ANOVA testing differences among sites and sources in seeds / reproductive shoot, proportion seeds reproductive, and seeds / m^2 (1999).

site	Tukey Grouping	source	Tukey Grouping
seeds per reproductive shoot:		*seeds per reproductive shoot:*	
Brown's	B	Brown's	A
Broad	B	Broad	B
Milford	A	Milford	A, B
Allen's	A	Allen's	A, B
proportion shoots reproductive:		*proportion shoots reproductive:*	
Brown's	B	Brown's	B
Broad	A	Broad	A, B
Milford	C	Milford	A
Allen's	C	Allen's	A, B
total seeds:			
Brown's	B, C		
Broad	C, D		
Milford	D		
Allen's	A		

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TABLE 14. Results of 2-way ANOVAs testing differences in measures of seedling vegetative fitness. P values ≤ 0.05 are in bold.

source (DF)	SS	MS	F	p	% variance explained
% germination:					
site (3)	26259.88	8753.29	38.66	**0.0001**	43.71
source (3)	153.08	51.03	0.23	0.8787	0.25
site*source (9)	1050.83	116.76	0.52	0.8615	1.75
error (144)	32607.60	226.44			54.30
seedling blade area/bottom:					
site (3)	10251.08	3417.03	54.89	**0.0001**	53.55
source (3)	129.54	43.18	0.69	0.5575	0.68
site*source (9)	419.52	46.61	0.75	0.6637	2.19
error (144)	8341.80	62.25			43.58
seedling biomass/m²:					
site (3)	0.1346	0.0449	42.52	**0.0001**	44.84
source (3)	0.0057	0.0019	1.79	0.1515	1.90
site*source (9)	0.0080	0.0009	0.84	0.5787	2.66
error (144)	0.1519	0.0011			50.60

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TABLE 15. Summary of ANOVA and RMANOVA results for Transplant I, Transplant II, and Seed Transplant experiments.

	figure(s)	site	source	site*source	higher fitness at native site?
Transplant I					
longest blade length	3A, 3B	*	*	*	yes (for Allen's source)
longest blade width	3C, 3D	*	*	n.s.	
longest blade area	4A, 4B	*	*	*	yes (for Allen's source)
vegetative density	4C, 4D	*	n.s.	*	yes
total biomass (1998)	5E	n.s.	*	n.s.	
total biomass (1999)	5F	*	*	n.s.	
seeds/reproductive shoot	6A, 6B	*	n.s.	n.s.	
reproductive shoots	6C, 6D	*	*	n.s.	
seeds/m²	6E, 6F	*	n.s.	n.s.	
Transplant II					
total blade length	7A – D	*	*	n.s.	
total blade width	7E – H	*	*	*	no
total blade area	8A – D	*	*	n.s.	
vegetative density	8E – H	*	*	*	no
total biomass	9C	*	n.s.	n.s.	
seeds/reproductive shoot	10A	*	*	n.s.	
reproductive shoots	10B	*	*	n.s.	
seeds/m²	10C	*	n.s.	n.s.	
Seed Transplant					
% germination	12A	*	n.s.	n.s.	
seedling blade area	12B	*	n.s.	n.s.	
seedling biomass	12C	*	n.s.	n.s.	

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Figure Legends

Figure 1
Map of Chesapeake Bay (Virginia, USA) indicating sites used in reciprocal shoot and seed transplants.

Figure 2
(A) Mean (± 1 SE) area of longest leaf blade per shoot per area of bottom, and (B) mean (± 1 SE) density of shoots per area of bottom for natural eelgrass populations at Brown's Bay, Broad Bay, Milford Haven, and Allen's Island. Measurements were made in July 1998 at sites used for transplants. Means bearing the same letter do not differ significantly ($p \leq 0.05$, Tukey test following significant 2-way ANOVA). n = 100 for each site. See Table 1 for ANOVA results.

Figure 3
Transplant Experiment I. Mean (± 1 SE) length of longest leaf blade per shoot at (A) Brown's Bay and (B) Allen's Island. Mean (± 1 SE) width of longest leaf blade per shoot at Brown's Bay (C) and Allen's Island (D). Transplants were measured at time of planting (initial single point), and biweekly monitoring began 4 months later (February). Dashed vertical lines show the times of peak biomass in 1998 and 1999, when reproductive characteristics were measured. n = 20 at each site for each time. See Table 3 for RMANOVA results.

Figure 4
Transplant Experiment I. Mean (± 1 SE) area of longest leaf blades per area of bottom at (A) Brown's Bay and (B) Allen's Island. Mean (± 1 SE) density of shoots at (C) Brown's Bay and (D) Allen's Island. Transplants were measured at time of planting (initial single point), and biweekly monitoring began 3 months later. Dashed vertical lines show the times of peak biomass in 1998 and 1999, when reproductive characteristics were measured. n = 20 at each site for each time. See Table 4 for RMANOVA results.

Figure 5
Transplant Experiment I. Mean (± 1 SE) (A, B) above-ground ash-free dry mass, (C, D) below-ground ash-free dry mass, and (E, F) total ash-free dry mass in 1998 and 1999. n = 20 at each site. See Table 5 for 2-way ANOVAs.

Figure 6
Transplant Experiment I. Mean (± 1 SE) seeds per reproductive shoot in (A) May 1998 and (B) May 1999. Mean (± 1 SE) proportion shoots reproductive in (C) May 1998 and

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(D) May 1999. Mean (± 1 SE) seeds per area bottom in (E) May 1998 and (F) May 1999. n = 20 at each site. See Tables 6 and 7 for 2-way ANOVA results.

Figure 7
Transplant Experiment II. Mean (± 1 SE) length of longest leaf blade per shoot at (A) Brown's Bay, (B) Broad Bay, (C) Milford Haven, and (D) Allen's Island. Mean (± 1 SE) width of longest leaf blade per shoot at (E) Brown's Bay, (F) Broad Bay, (G) Milford Haven, and (H) Allen's Island. Transplants were measured at time of planting (initial single point), and biweekly monitoring began 3 months later. Dashed vertical line shows the times of peak biomass in 1999, when reproductive characteristics were measured. n = 20 at each site for each time. See Table 7 for RMANOVA results.

Figure 8
Transplant Experiment II. Mean (± 1 SE) area of all leaf blades per area bottom at (A) Brown's Bay, (B) Broad Bay, (C) Milford Haven, and (D) Allen's Island. Mean (± 1 SE) density of shoots per area bottom at (E) Brown's Bay, (F) Broad Bay, (G) Milford Haven, and (H) Allen's Island. Dashed vertical line shows the time of peak biomass in 1999, when reproductive characteristics were measured. Horizontal dashed line shows initial planting density. n = 20 at each site for each time. See Table 8 for 2-way RMANOVA results and Table 9 for Tukey results.

Figure 9
Transplant Experiment II. Mean (± 1 SE) above-ground (A), below-ground (B), and total (C) ash-free dry mass in 1998 and 1999. n = 20 at each site. See Table 10 for 2-way ANOVA results.

Figure 10
Transplant Experiment II. Mean (± 1 SE) (A) seeds per reproductive shoot, (B) proportion shoots reproductive, and (C) seeds per area bottom at Brown's Bay, Broad Bay, Milford Haven, and Allen's Island in May 1999. n = 20 at each site. See Table 11 for 2-way ANOVA results and Table 12 for Tukey results.

Figure 11
Seed Transplant. Mean (± 1 SE) dry mass of individual seeds from Brown's Bay, Broad Bay, Milford Haven, and Allen's Island in May 1999. Means bearing the same letter do not differ significantly ($p \leq 0.05$, Tukey test following significant 2-way ANOVA). n = 10 for each source.

Figure 12
Seed Transplant. Mean ± 1 standard error (SE) (A) percent germination, (B) total blade area / bottom area, and (C) seedling biomass per bottom area at Brown's Bay, Broad Bay,

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Milford Haven, and Allen's Island in May 2000. $n = 10$ for each site. Sites bearing the same letter do not differ significantly ($p \leq 0.05$, Tukey test following significant 2-way ANOVA).

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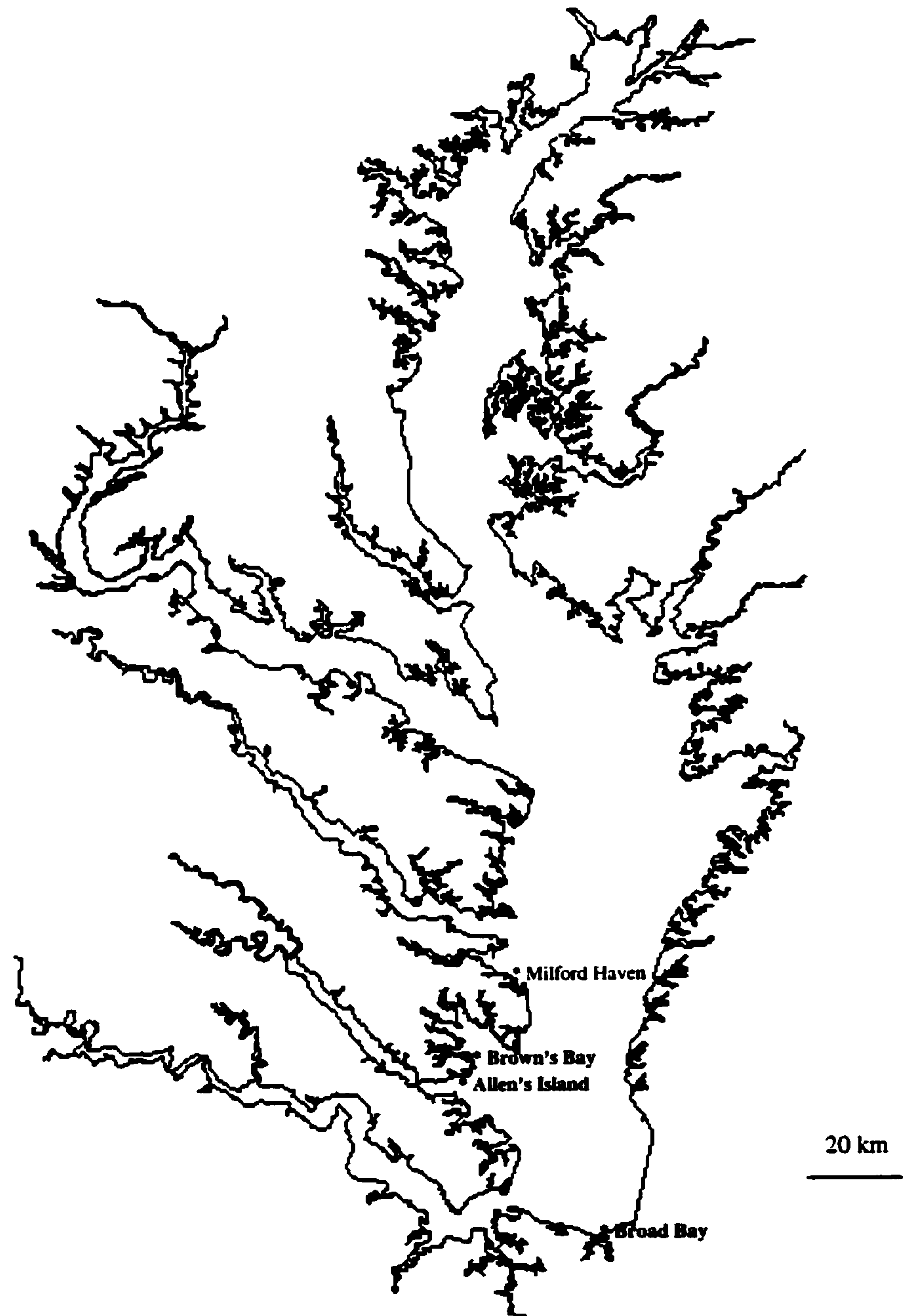
Figure 1



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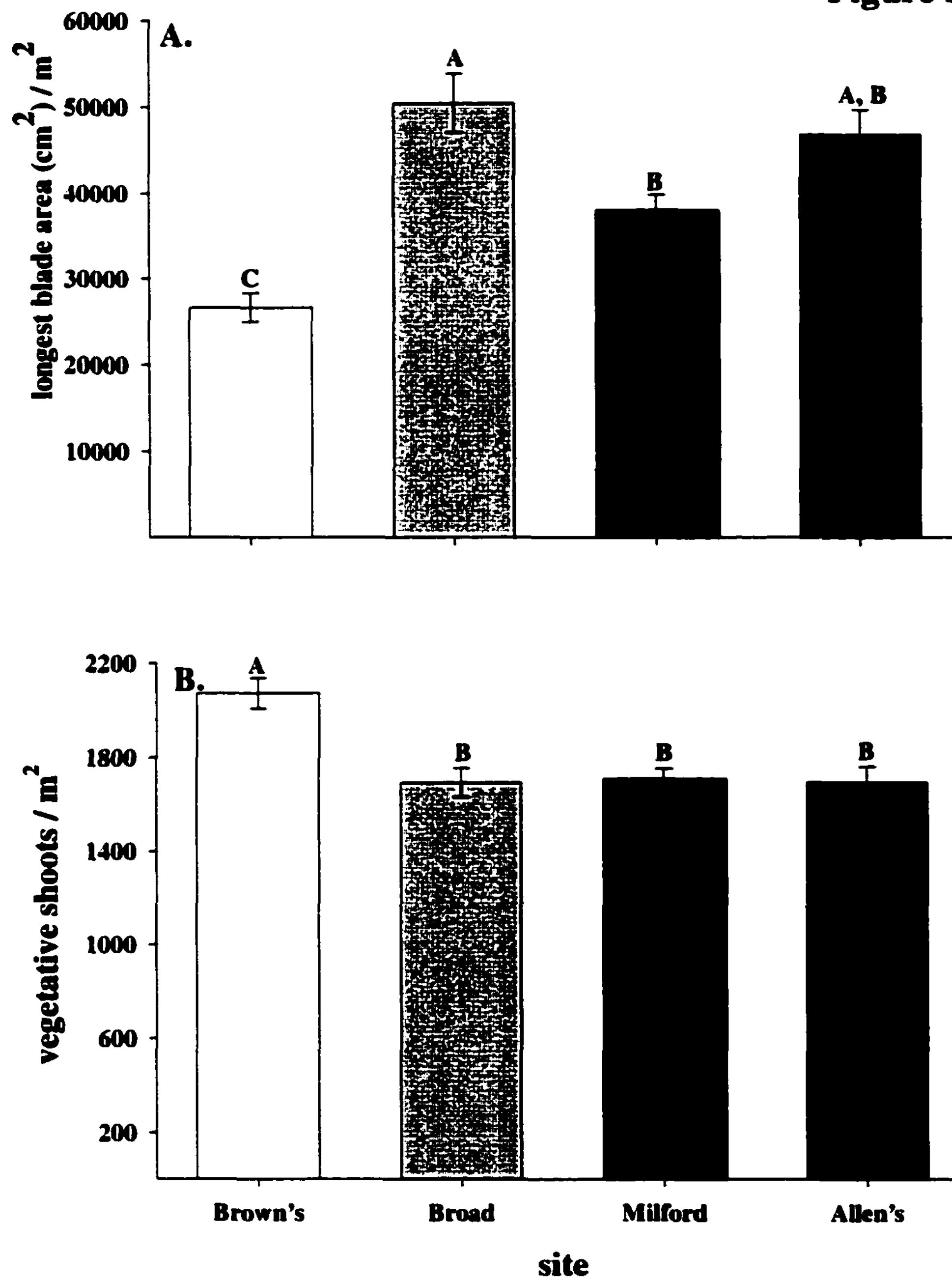

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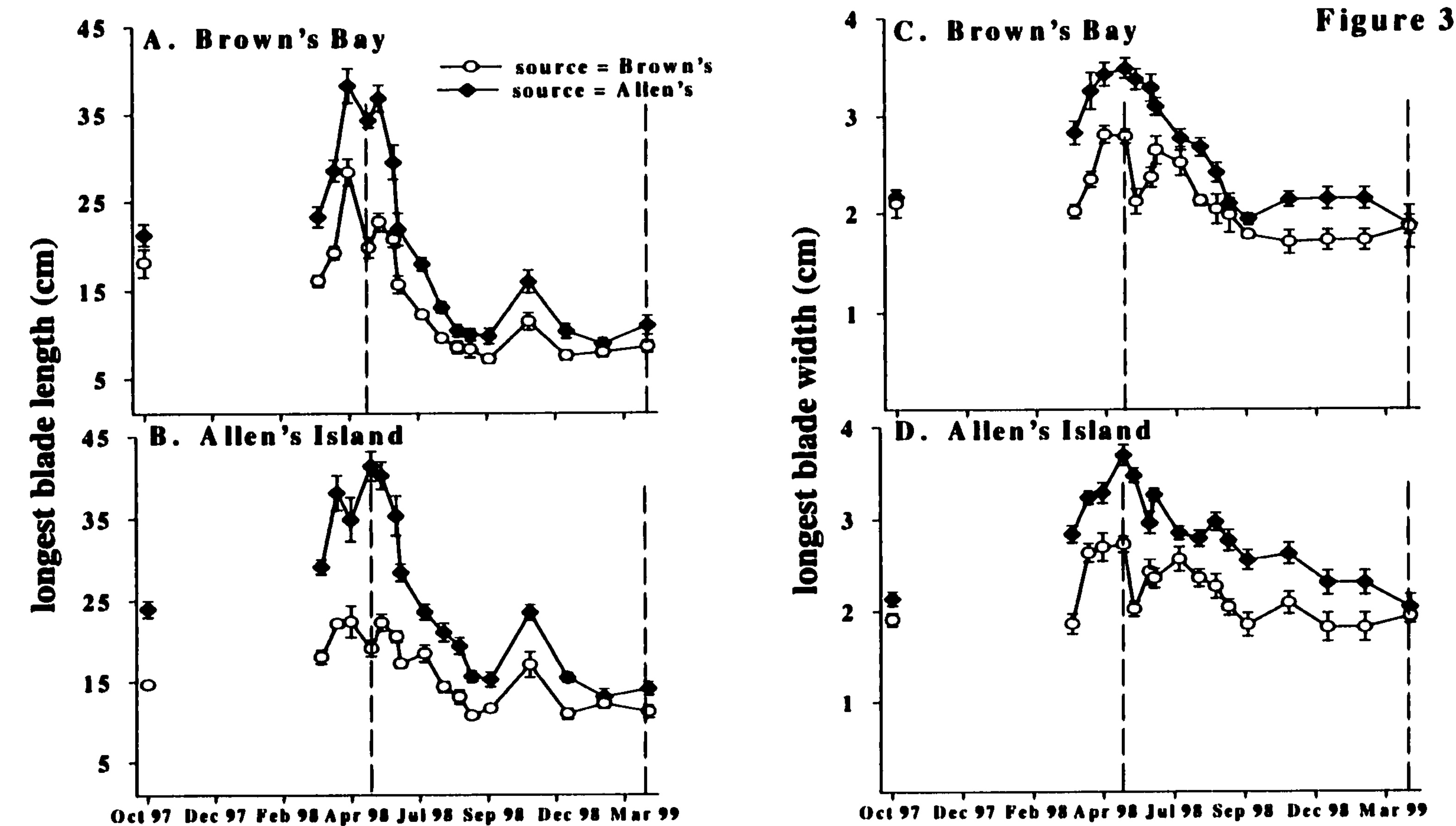

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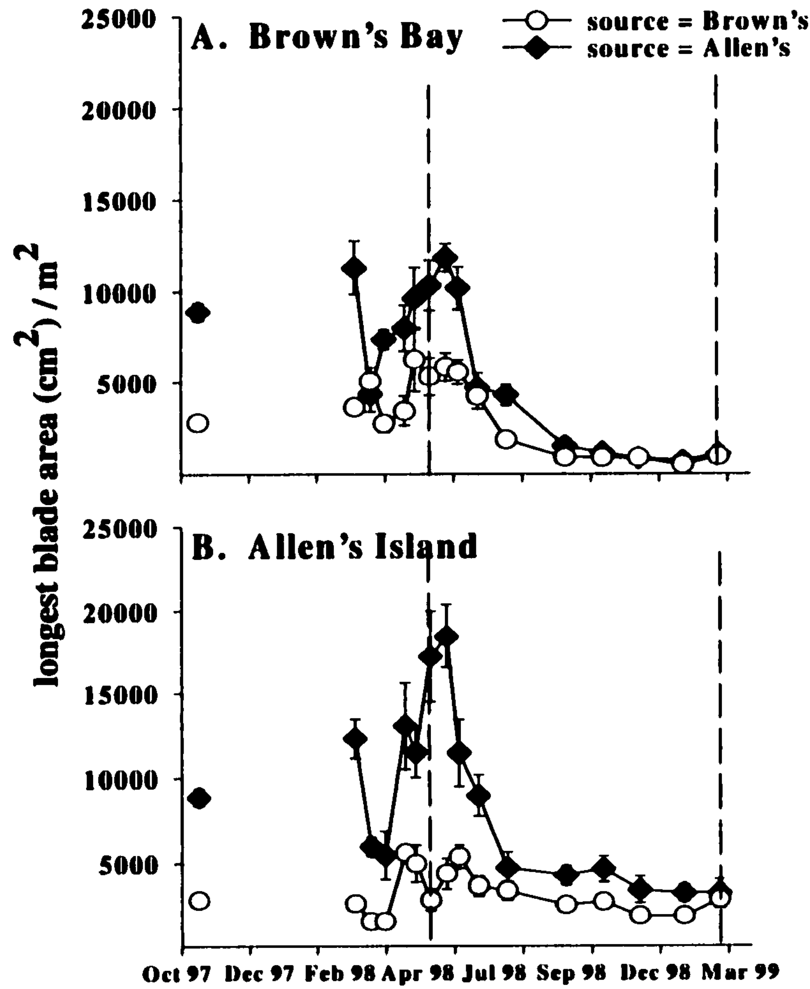
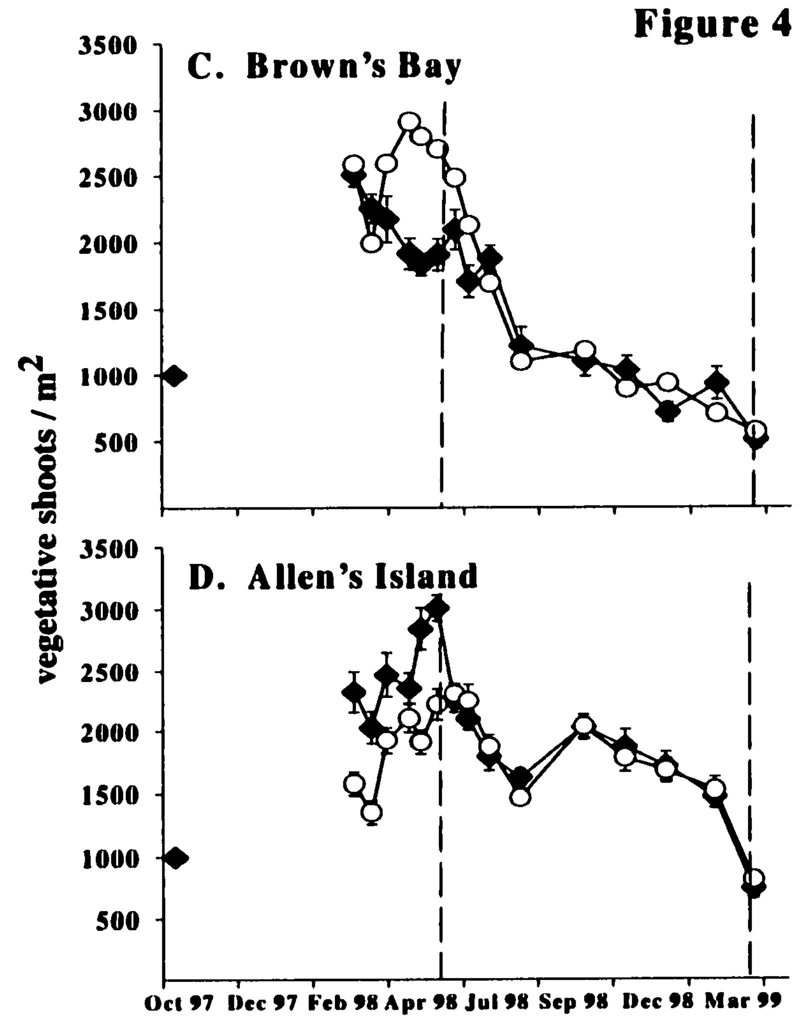
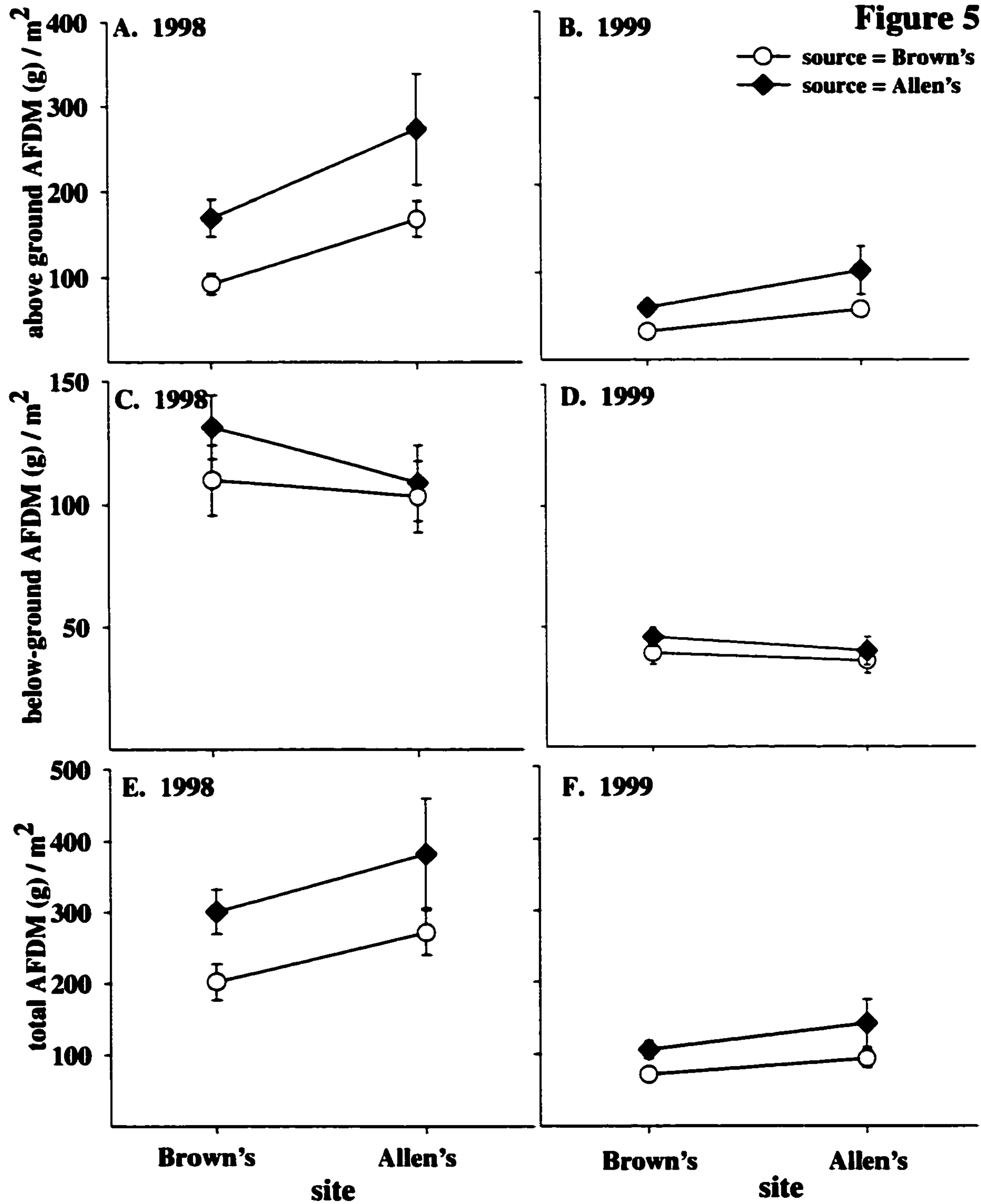
Figure 4







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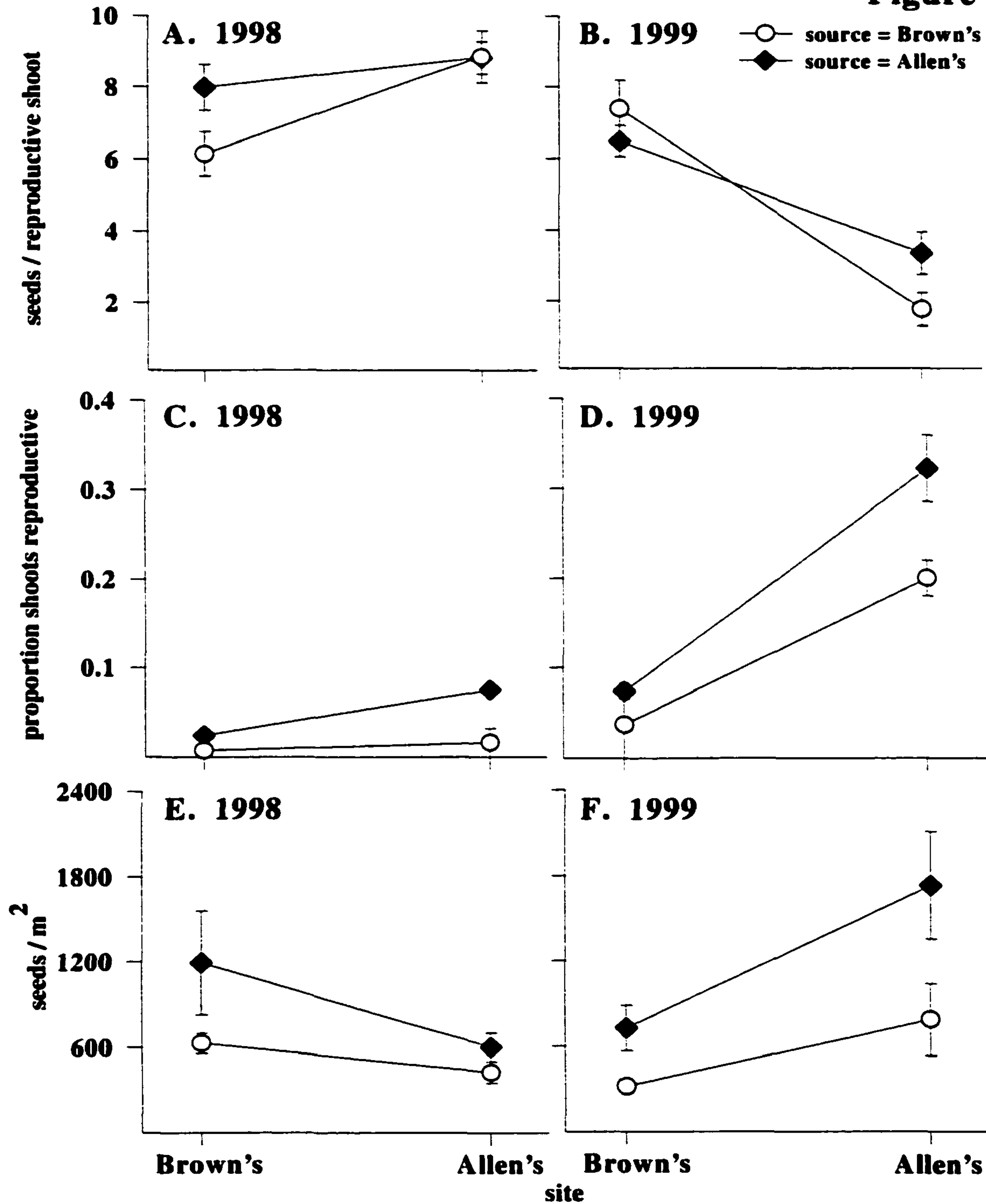

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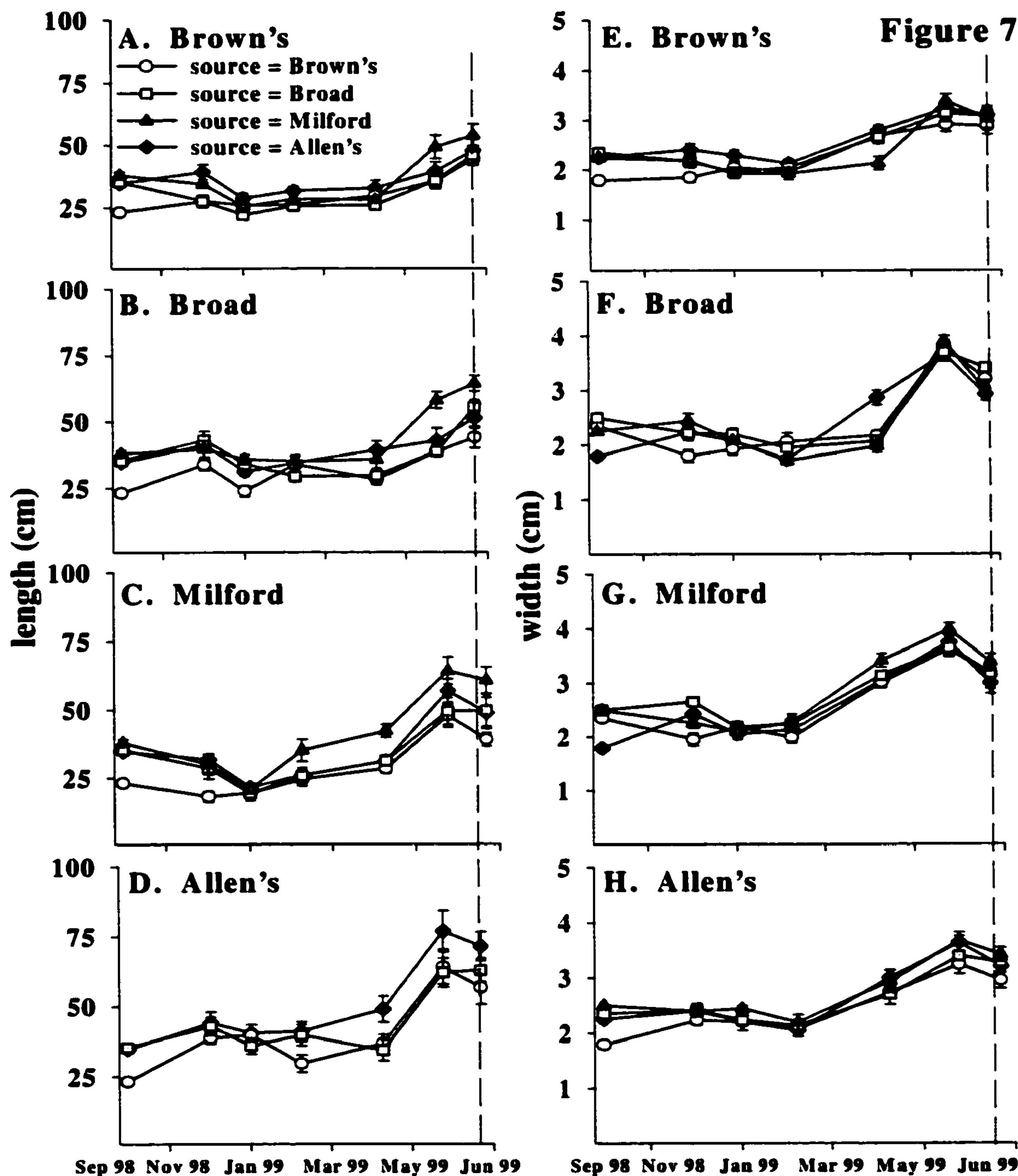

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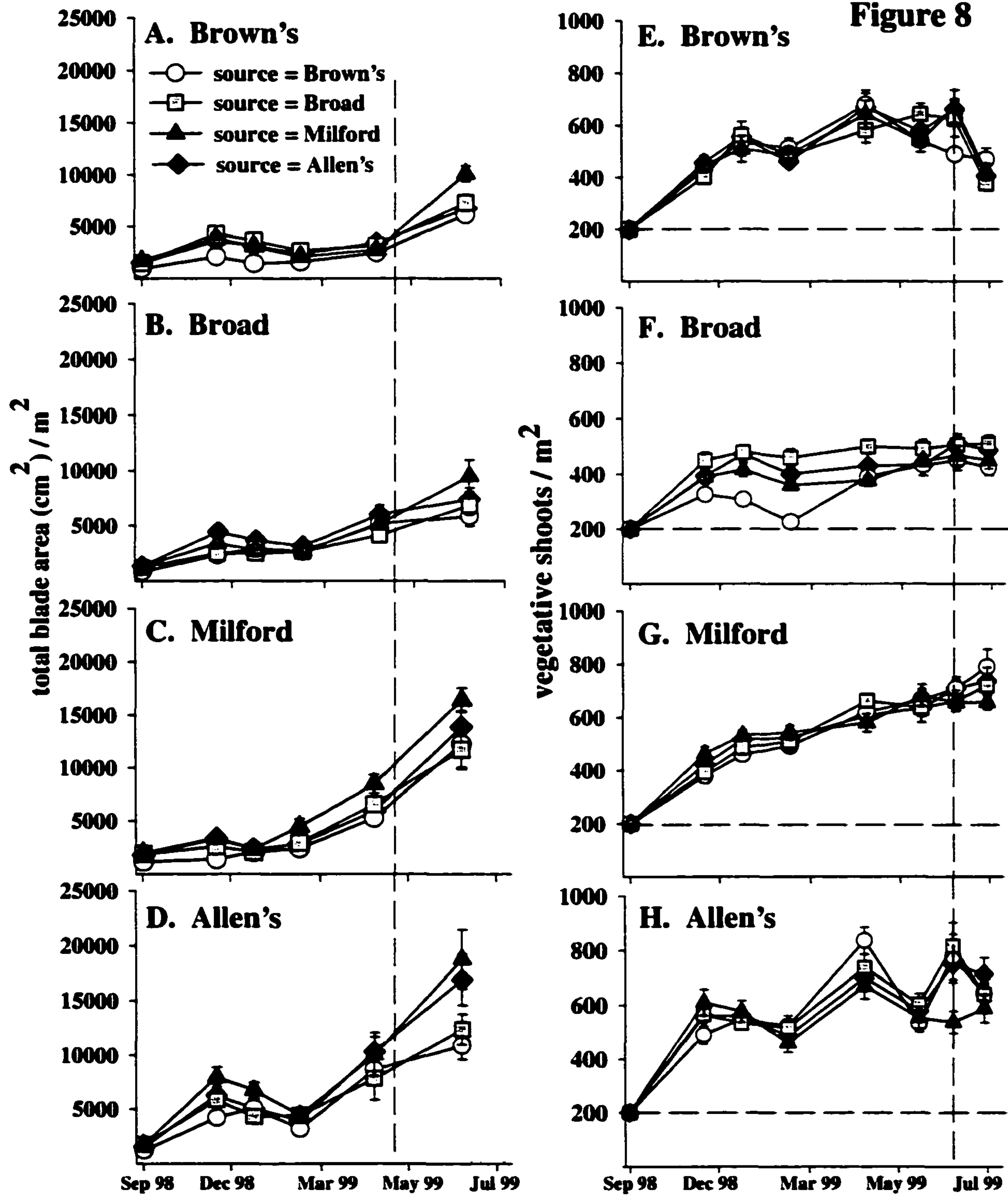

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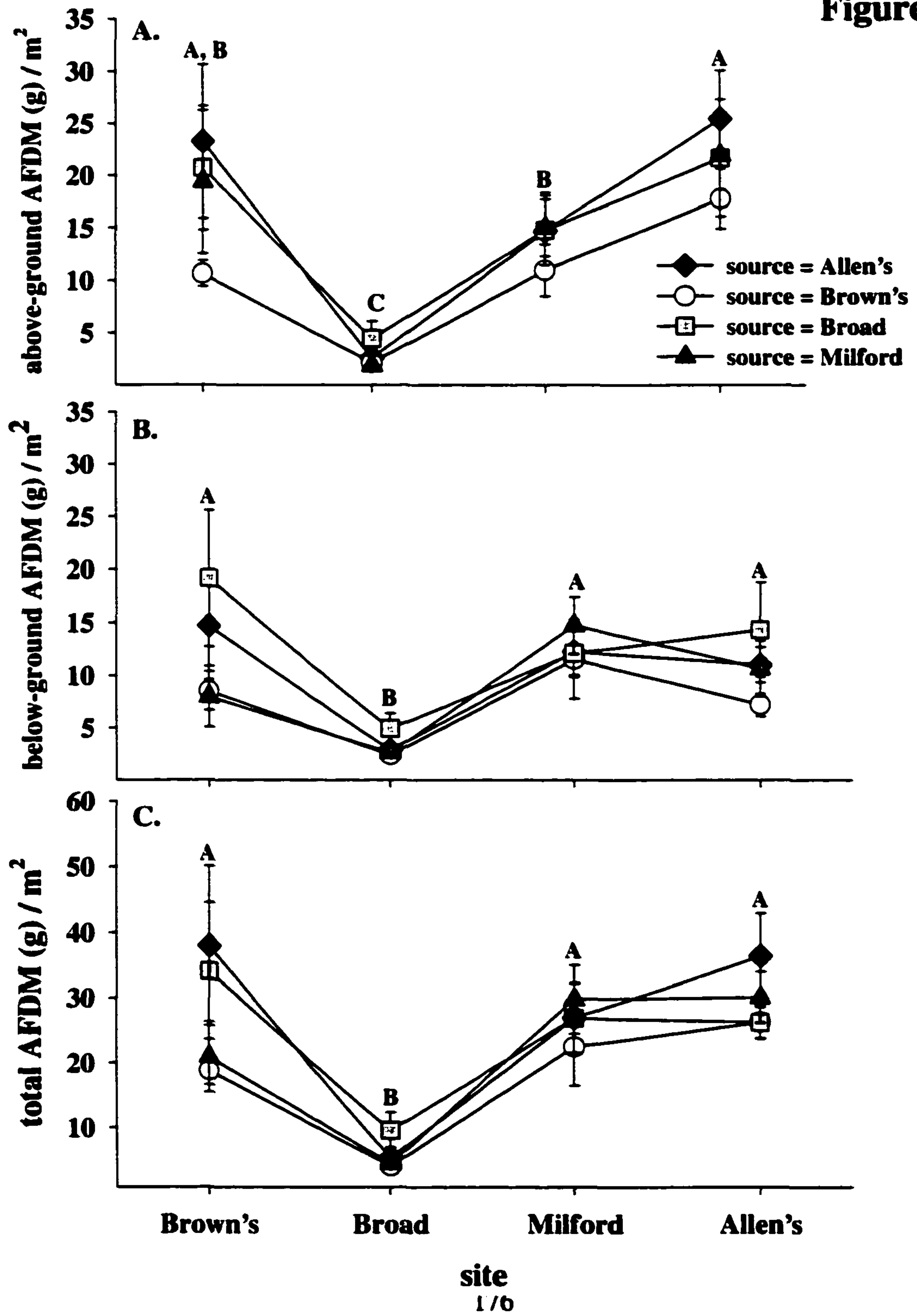

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Figure 10

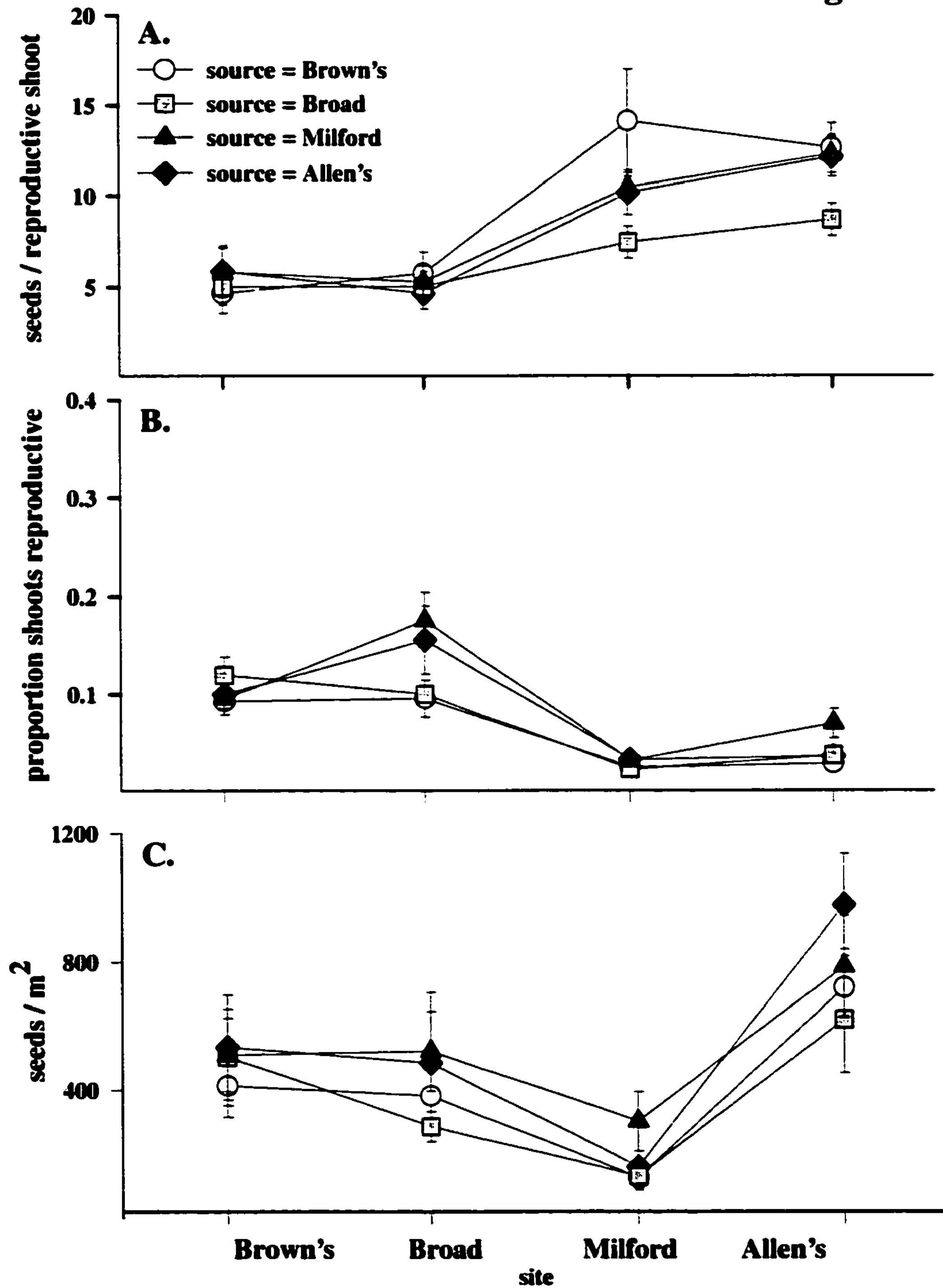

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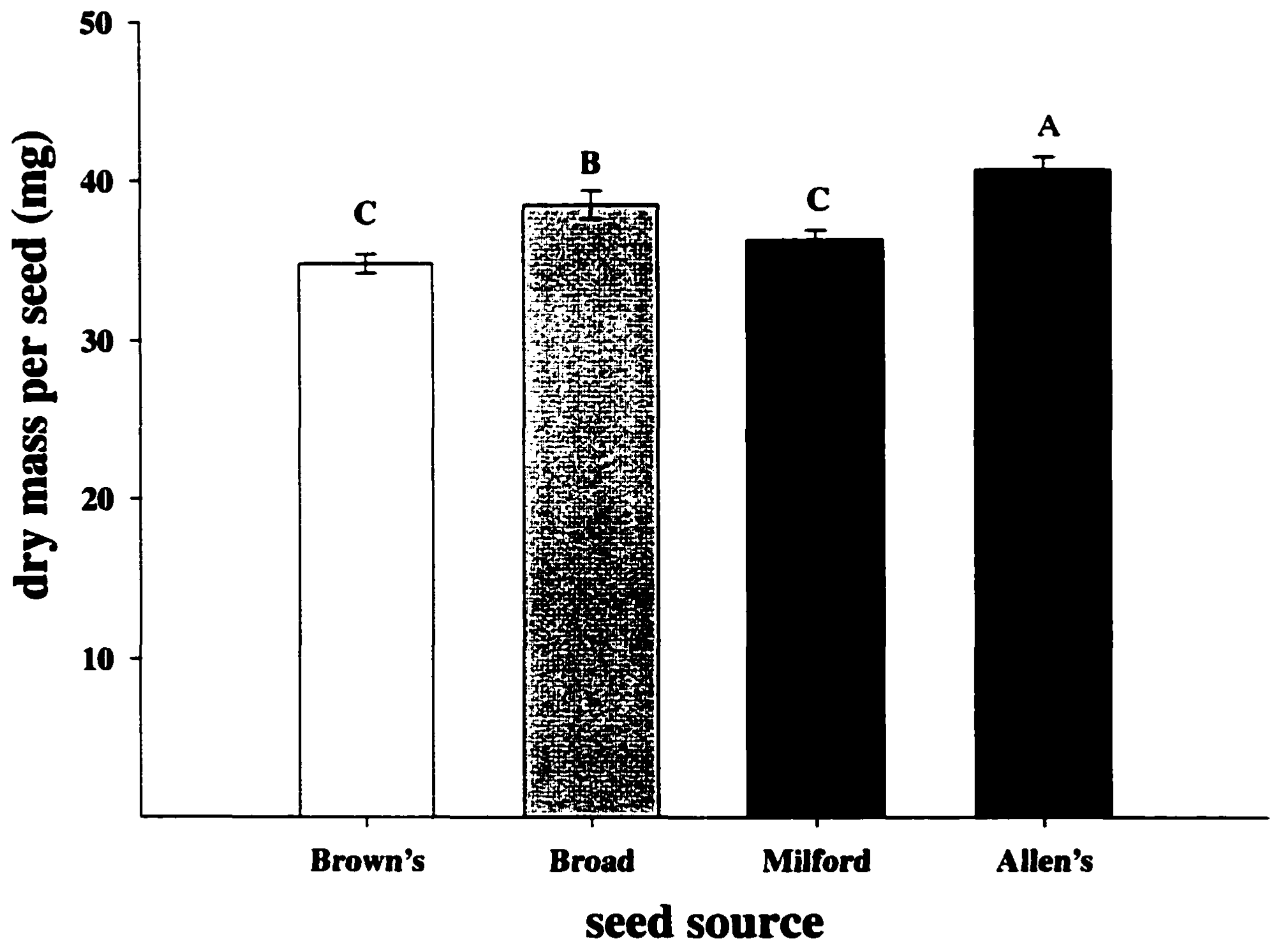
Figure 11



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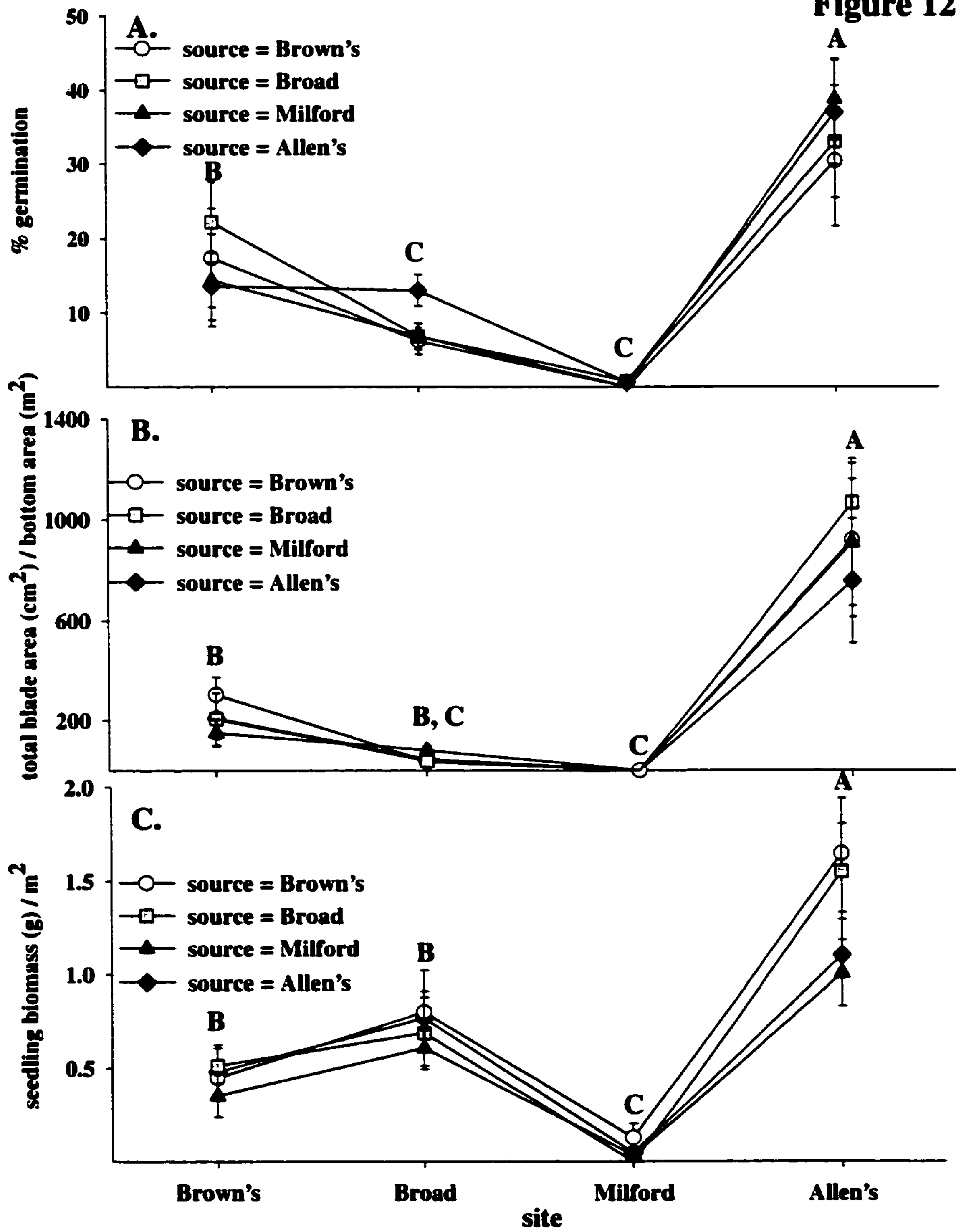

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Chapter 5: Conclusions

Problems Addressed

This dissertation addressed the linkages between population genetic structure, mating systems, and phenotypic adaptation in Chesapeake Bay eelgrass. First, it examined the distribution of genetic diversity in Chesapeake Bay and looked for connections between this diversity and demographic variables. Next, it examined the mating structure of Chesapeake Bay eelgrass. Finally, it looked for evidence of local adaptation in these eelgrass beds. The data presented here contribute to the growing understanding of Chesapeake Bay eelgrass and provide a more comprehensive picture of the demographic and genetic processes affecting these populations.

Summary of Research Results

Levels and patterns of genetic diversity within old (> 65 years) beds were hypothesized to be higher than those in young (< 7 years) beds, which were probably founded by few genetic individuals. Likewise, diversity levels were expected to be higher in large (>100 ha) than in small (< 10 ha) beds. This would not be the case, though, if eelgrass' long-distance dispersal capabilities were realized on a regular basis, and genetic structure was homogenized across demographic lines. The genetic survey presented in this dissertation, which used twelve Chesapeake Bay eelgrass beds and seven allozyme loci, revealed strong subdivision among beds and inbreeding within beds. This genetic subdivision presumably reflects low gene flow resulting from restricted pollen and seed dispersal in this species, as shown in some previous studies. There were

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no significant differences in any of the population genetic parameters measured as a no effects of age or size on measures of genetic diversity. The only exception was F_{IS}, the inbreeding coefficient, which suggested higher levels of inbreeding in old, small beds.

Several factors contributed to the lack of bed age and size effects. First, estimates of population age and size might have been inaccurate, or the endpoints of the age and size continuum might have been on scales too coarse to capture processes important to Chesapeake Bay eelgrass populations. For instance, it is likely that population processes in 10 and 100 ha beds are comparable, but processes in beds less than 1 ha are more dependent on founder effects, genetic drift, and clonal competition that might influence the measured genetic diversity parameters. Another not mutually exclusive possibility is that gene flow via seed dispersal, though relatively rare, is sufficiently frequent to maintain genetic diversity and obscures patterns that might be expected to vary with age and size.

The second component of this research involved laboratory mating experiments. In this study, seed set from selfed, inbred and outbred matings was hypothesized to be similar since spatial and temporal considerations (protogyny, dispersal limitation) which could dictate mating patterns were overcome by the design of this study. In fact, hand-pollinations revealed not only that Chesapeake Bay eelgrass can self-fertilize, but also that self-fertilization produced more seeds than within-bed or outcrossed matings. Therefore, it appears that Chesapeake Bay eelgrass is adapted to extremely localized breeding (selfing). Inbreeding is probably common in natural populations of this clonally spreading plant, and this study revealed no discernable inbreeding depression. Though

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self-fertilization within an inflorescence might be uncommon *in situ*, self-fertilization could occur among inflorescences or among clonally-produced shoots; these are demographically distinct processes which yield the same genetic consequences. Production of seeds via inbreeding, along with clonal spread, probably reinforces the patchy genotype distribution within beds and contributes to the consistently high inbreeding coefficients (F_{IS}) found.

The third component of this dissertation explored the basis for the extensive phenotypic variation seen in Chesapeake Bay eelgrass. Because *Zostera marina* has high intrinsic levels of phenotypic plasticity, Chesapeake Bay eelgrass was not expected to show strong evidence of local genetic adaptation. In the first transplant experiment, data for shoot density and, to a lesser extent, seed production, both of which are good predictors of eelgrass transplant success (Williams 2001), showed a home-site advantage consistent with local adaptation. However, other measures of eelgrass performance and fitness did not support local adaptation, and existing evidence for adaptation was inconsistent among years and sites. Moreover, temporal and site variation in eelgrass performance was generally stronger than source effects. Thus, it appears that most responses of Chesapeake Bay eelgrass to changing environmental conditions are rooted in the extensive phenotypic plasticity previously documented in this species (Phillips *et al.* 1983, Backman 1991).

Conclusions

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Chesapeake Bay *Zostera marina* beds show strong population structure. These beds contain high levels of morphological and genetic diversity, which are partitioned such that beds maintain considerable phenotypic and genotypic distinctness. Though beds show evidence of strong inbreeding (depressed heterozygosity levels), they retain substantial within-bed genetic diversity. This likely results from pockets of local breeding and/or clonal growth within a bed, such that loss of heterozygosity occurs in small clusters but the entire bed retains a mosaic of distinct clones and closely related genotypes. Despite strong genetic differentiation among beds, there was no evidence for isolation by distance, either within or among beds. Inbreeding, clonal growth, or restricted dispersal might contribute to the observed lack of relationship between genetic and geographic distance. Consistent with the evidence for regular inbreeding in Chesapeake Bay eelgrass, greenhouse experiments revealed no inbreeding depression; in fact, there was highest fitness in selfed matings. These data suggest that Chesapeake eelgrass is genetically adapted to the highly localized breeding implied by the genetic structure of populations. This sort of mating pattern is expected in plants from stressful or unpredictable environments, as in the estuarine environment of *Z. marina*. In Chesapeake Bay eelgrass, protogyny seems to be an evolutionary remnant rather than a mechanism for preventing self-fertilization.

A question of major interest for both basic and applied ecology is what controls the extensive phenotypic variance among beds of Chesapeake Bay eelgrass. Previous work (Backman 1991) suggested that phenotypic diversity reflected high levels of intrinsic plasticity in this plant. In the transplant experiments presented here, source

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morphology and growth characteristics were often retained for considerable periods (up to 10 months) at new sites consistent with genetic control. However, most phenotypic measures eventually converged such that transplants closely resembled local plants. The time course of these trends suggests considerable canalization, but strong phenotypic plasticity, in morphological and performance traits of Chesapeake Bay eelgrass shoots.

Phenotypic variance among and within eelgrass beds could also be affected by population genetic structure. Allozyme diversity levels in Chesapeake Bay eelgrass beds were higher than allozyme diversity levels in California beds (Williams and Davis 1996), and DNA-based measures would presumably reveal even higher genetic diversity in Chesapeake populations. Perhaps surprisingly, however, heterozygosity levels reported here, though lower than expected from allele frequencies (i.e., high F_{IS} values), were equivalent to or higher than heterozygosity levels reported in a microsatellite study of Baltic Sea eelgrass populations (Reusch 2002). The relatively high frequency of sexual reproduction in Chesapeake Bay eelgrass (Silberhorn *et al.* 1983) might explain the retention of high heterozygosity relative to eelgrass in some other regions. High levels of allozyme diversity in Chesapeake *Z. marina* could also be fostered by very limited dispersal, which allows individual beds to maintain numerous unique genotypes, or of frequent disturbance, which limits spread of individual clones.

Clearly, multiple processes create genetic structure in Chesapeake Bay eelgrass beds. Establishment of beds by small numbers of founders, followed by genetic drift and clonal competition, are probably important in creating initial genetic structure in the smallest or newest beds and in establishing the strong subdivision among beds.

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However, this study found little evidence that such processes leave a signature that differs among beds of the ages or sizes tested here. Though seeds (when attached to a reproductive shoot) are able to move large distances, and they may be invaluable at helping beds recover following environmental catastrophe (Harwell and Orth 2002), the genetic data presented here suggest that the contribution of dispersing seeds (or adults) to established beds is probably quite small. That is, there is an important distinction between potential dispersal and realized gene flow in Chesapeake eelgrass beds.

Non-random mating, in addition to the low gene flow that produces subdivision among beds, contributes significantly to genetic diversity and structure in Chesapeake Bay eelgrass beds. Inbreeding and selfing are biologically possible in this species, had no discernable negative fitness impact in these experiments, and, based on genetic data presented here, occur frequently in Chesapeake eelgrass beds. Vegetative growth very likely also contributes to the observed heterozygote deficiencies, through a Wahlund effect in which clones might be multiply sampled. Overall, localized selection seems to have had little impact on bed genetic structure, at least at the scales examined here. Chesapeake Bay eelgrass showed evidence of local adaptation in shoot density and, to a lesser extent, seed number in the first experiment. These parameters are considered quite important in measuring transplant success (Williams 2001), and they are likely also important to ecosystem function (Hemminga and Duarte 2001). Therefore, local adaptation, though weak, could be significant. It is noteworthy, however, that no response variable showed significant home-site advantage in the more comprehensive second transplant experiment. Overall, data from both transplant studies seems to

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support a dual role for plasticity (stronger) and local adaptation (weaker) in Chesapeake Bay eelgrass.

Implications for Restoration and Management of Chesapeake Bay Eelgrass

The genetic structure of Chesapeake Bay eelgrass beds reveals that beds generally contain substantial amounts of genetic diversity and that beds differ strongly in genetic composition. Therefore, conservation of maximal genetic diversity in Chesapeake Bay eelgrass would argue that no bed be completely destroyed. On the other hand, the phenotypic plasticity documented in this dissertation implies that the site from which Chesapeake Bay eelgrass plants are taken for transplant is much less crucial that the site into which they are planted. Canalization of adult shoots can be overcome within a single year, and shoots grown from seeds have morphologies more similar to other plants in their growing site than to those from their source. Thus, the strong phenotypic plasticity of Chesapeake Bay eelgrass should enhance prospects for eelgrass restoration in a variety of environments. At the same time, the role of genetic diversity in enhancing eelgrass performance (Williams 2001), which was not explicitly tested in this study, should always be considered when creating conservation restoration strategies.

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