AN ABSTRACT FOR THE DISSERTATION OF

Sean E. Matson for the degree of Doctor of Philosophy in Animal Science, presented on June 1, 2010.

Title: Development, Evaluation and Application of a Mixed-family Selective Breeding Method for the Pacific Oyster (*Crassostrea gigas*)

Abstract approved:_____

Howard H. Meyer

This dissertation develops mixed family selection for Pacific oysters using marker-based pedigree reconstruction. It focuses on improving the efficiency of parentage assignment, determining the optimum life stage to mix oyster families for rearing and selection, comparing mixed-family and separate-family selective breeding in the field, and applying the mixed method to estimate the heritability of shell shape. We developed novel computer software, P-LOCI (available at http://marineresearch.oregonstate.edu/genetics/PLOCI.html), which identifies the most efficient set of codominant markers for assigning parentage, accounting for marker linkage, mating design, null alleles and genotyping error, and found that the most efficient group of loci for assignment is not necessarily comprised of the top individually ranked loci, or best for all populations. We determined the optimum time to mix oyster families for rearing and selection in the field; overall, planting size is the most prudent time to mix families for MFS, due to high variability in family representation produced during larval and nursery stages. Mixing families at stages earlier than this for selection on field traits would require

pre-planting genotyping of large samples for estimation of initial family representation, which would add substantial cost, or other special considerations. Rearing mixed family groups of oysters in the field yielded very similar results to rearing the same families separately, (r = 0.817 for two-site average individual weight at harvest), demonstrating it is unlikely associative effects are of great importance in the Pacific oyster. Our results show that the mixed method was well-suited for individual traits and walk-back selection, but would incur higher costs than the separate method to estimate survival with lower precision. Finally, we utilized the mixed method to estimate the heritability of shell shape using midparent-offspring regression; we estimated shell depth heritability as 0.404 ± 0.14 and shell width as 0.287 ± 0.11, nearly equal to the only other study for the Pacific oyster, demonstrating potential for selective breeding on these traits in this U.S. population, and similar results between methods. Overall, we found that mixed-family rearing is viable for Pacific oyster breeding, given some important restrictions.

Development, Evaluation and Application of a Mixed-family Selective Breeding Method for the Pacific Oyster (*Crassostrea gigas*)

by
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APPROVED:

Major Professor, representing Animal Science

Head of the Department of Animal Science

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Sean E. Matson, Author

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CONTRIBUTION OF AUTHORS

Dr. Michael Banks contributed to the planning and development of P-LOCI software (Chapter 2). Will Eichert wrote the code for P-LOCI. Dr. Chris Langdon was the Principal Investigator on the grants for Chapters 2 through 5, and contributed to the planning and development of Chapters 4 and 5. Dr. Mark Camara contributed to the planning of P-LOCI software, provided comments on the P-LOCI manuscript, contributed to Chapter 1, contributed to planning for Chapters 3 and 4, and was Co-Principal Investigator on the grant for Chapters 2 through 4.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

Figure Page

LIST OF TABLES

LIST OF TABLES (continued)

CHAPTER 1. GENERAL INTRODUCTION

Selective breeding in bivalve shellfish is complicated by their somewhat peculiar life-histories compared to most agricultural species. Williams (1975) argued in his "Elm-Oyster Model" that these high-fecundity species are much more dependent on sexual reproduction and the genetic diversity it generates than low-fecundity species because intense selection at early life history stages in a dynamic, heterogeneous environment strongly favors genetic variation; the enormous number of cell divisions required to produce hundreds of millions of gametes over a lifetime results in a high genetic or mutational load of deleterious recessive alleles. In hatchery-spawned oysters, this genetic load combined with high variance in reproductive success, has the potential to produce severe inbreeding depression in just a few generations (Bierne et al. 1998, Evans et al. 2004, Launey and Hedgecock 2001). If not accommodated for, it would severely limit the long-term viability of selective breeding (Falconer and Mackay 1996, Lynch and Walsh 1998, Newkirk 1978). The current major publicly funded selective breeding effort for Pacific oysters in the United States, the Molluscan Broodstock Program (MBP), has controlled inbreeding by producing 2-3 groups of 60 full-sib families each year, rearing each family separately from spawn to harvest and then selecting primarily among families to identify broodstock for the next generation (Langdon et al. 2003). While this approach is effective in limiting inbreeding and generating additive genetic change, it also has some potential for undesirable side-effects. Evaluating oysters grown in family-specific bags confounds the additive genetic effects targeted by a breeding program with other effects including small-scale environmental heterogeneity (oyster position within

the growing unit), interactions among genotypes within a growing unit, and unit-specific and family-specific density effects due to differential mortality. While blocked and replicated experimental designs can statistically randomize and/or correct for the environmental effects at spatial scales larger than the growing unit (Sokal and Rohlf 1996), this is labor intensive and costly. Typically, MBP field trials employ 480 individual grow-out bags or lantern net compartments (Langdon et al. 2003). This, together with the 60 individual units in the hatchery and nursery required to produce these separate families for the field trials, make for a large investment in labor, equipment and tideland resources. Without public or cooperative funding, selective breeding of oysters is only possible for growers or hatchery operators with high capital, which is atypical of shellfish farmers in the current industry condition of the Pacific coast of the United States.

An alternative strategy is to tag and mix individuals from a large number of families so that they can be reared together and to sort the superior animals by family at harvest. This strategy would work to homogenize the effects of environmental variation, genetic interactions and density among families. Theoretically, this could enable an increase in selection intensity for some traits, and economization of seed production and field trials, potentially making them smaller, cheaper and more informative. Mixed family selection lends itself to individual selection, which harnesses both within and among-family additive genetic variation for genetic improvement. The major barrier to this approach in oysters is that physically tagging microscopic larvae and tiny juveniles is currently infeasible; the technology for this application is currently unknown. Our

experience indicates that larger juveniles of planting size (10mm shell length), can be individually tagged with difficulty, but rearing juveniles to this size in the nursery incurs a significantly higher cost than typical planting size (3-4mm), and tags are frequently shed due to abrasion and barnacle growth, and must be reapplied within months (C. Brooks, pers. comm.). Additionally, it would be prohibitive to physically tag more than a few thousand animals, which would limit genetic progress.

Modern molecular genetic methods, however, can circumvent these limitations. Highly polymorphic DNA markers make sorting mixed animals of different parentage possible without physically tagging them during the rearing phase (Blouin 2003, Jones and Ardren 2003). Individual genetic marker loci provide information about relatedness roughly in proportion to the number of alleles segregating in the study population (Ritland 2000), so the best markers for these purposes are highly polymorphic, such as microsatellite markers. Single nucleotide polymorphisms or SNPs are also frequently applied for parentage determination, although 10 to 20 times as many SNPs are usually needed as microsatellites to make the same assignments, since most SNPs are biallelic, and they carry four alleles at most (Anderson 2005). Both of these marker types produce results that are reproducible among different crosses, populations, and laboratories. More than 100 microsatellite markers have been developed and mapped in Pacific oysters (Hedgecock et al. 2003, Hedgecock et al. 2004, Hubert and Hedgecock 2004, Huvet et al. 2000, Li et al. 2003, Li and Guo 2004, Magoulas et al. 1998, McGoldrick et al. 2000, Sekino et al. 2003), while there are

currently not enough suitable Pacific oyster SNPs to enable parentage determination, which makes microsatellites the logical choice for this study. Although other PCR-based markers, such as random amplified polymorhic DNA (RAPD) and amplified fragment length polymorphisms (AFLP), are less expensive to develop and have been used to determine parentage (Gerber et al. 2000), they are specific to the population or crosses in which they are developed, which limits their usefulness.

Microsatellite markers have been successfully used to determine parentage in a wide variety of plant and animal species. In aquaculture species, high parental assignment rates have been achieved in rainbow trout (Fishback et al. 2002, Herbinger et al. 1995), cod (Herbinger et al. 1997), Atlantic salmon (Herbinger et al. 1999), carp (Vandeputte et al. 2004), and red seabream (Perez-Enriquez et al. 1999) with only 3-6 microsatellite loci. Multiple families of European lobster have been reared together for selection and breeding and then assigned to parents with a 93.7% success rate using six microsatellite loci (Jorstad et al. 2005). Researchers in France have demonstrated that high parental assignment rates can be achieved in Pacific oysters with only three microsatellites when the parents are chosen to simplify the analysis (Taris et al. 2005), but choosing parents specifically to simplify the assignment problem is not compatible with selective breeding based on phenotypic superiority.

What remains to be seen, however, is whether mixed family selection will be cost-effective for use in a breeding program. At one extreme, the most powerful and most costly approach would be to obtain pedigree information for the entire

pre-selection population, to apply animal models to estimate breeding values of all potential parents (Henderson and Quaas 1976, Lynch and Walsh 1998), and to choose parents based on the degree to which they can be expected to contribute to improving the next generation, while minimizing relationship among crossed individuals. This is now the standard approach in large animal breeding where pedigree information is freely available from breeding records (Lynch and Walsh 1998). However, using genetic markers to obtain this level of detail is prohibitively costly for this to be practical in oysters, due in part to their high fecundity.

The other extreme would be a simple program of purely phenotypic selection (i.e. mass-selection) on collectively-fertilized mass spawns that use large numbers of parents with subsequent genotyping and parentage assignment of only a very small number of the most superior animals strictly to control inbreeding. This approach, however, is also likely to fail in oysters because high variance in reproductive success in uncontrolled mass spawns makes it very likely that a small number of parents will be highly overrepresented in the offspring population, leading to uncontrolled inbreeding in the next generation. Researchers have demonstrated that if no measures are taken to equalize family sizes, larval cultures mixed immediately post-fertilization produce seed oyster populations that are numerically dominated by a very small number of genetic families (Taris et al. 2005), due to differential fertilization success. We do not know, however, whether equalizing family representation at some point after fertilization, but during the early life cycle can correct this problem or if it will be necessary to rear families

separately all the way to the planting stage. What is required, therefore, is a workable strategy of controlled spawning and selective genotyping that falls somewhere between these two extremes.

Turning to the issue of how best to conduct selective genotyping in order to reduce cost, perhaps the simplest approach is what Doyle and Herbinger (1994) have termed "walk-back selection." Although this strategy, as presented by Doyle and Herbinger, involves grading or measuring all of the animals in the potential broodstock population, it calls for genotyping and assigning animals systematically starting from the most desirable tail of the distribution and walking back toward the population mean. An iterative procedure that combines phenotypic and pedigree information is then used to identify an unrelated group of superior broodstock to produce the next generation. A brief and simplified explanation is the following; the individual with the highest trait value or index value is selected as a replacement, but the second-ranked animal is only included if it is from a different family than the first. Similarly, the third is included only if it is from a family different from the first two. This process is repeated until replacements from a sufficient number of families to avoid inbreeding is obtained. In practice, it could be done in lots. This strategy could, however, fail if a large majority of the animals in the desirable tail of the distribution come from a very small number of families that are vastly superior to all others for the trait under selection (Doyle and Herbinger 1994). It could also fail in oysters of the genus *Crassostrea* due to severely skewed sex ratio, or irregular gonadal conditioning, both of which sometimes occur in this sequential hermaphrodite. Only practical

experience will tell whether these are fatal complications, but computer simulations suggest that they are probably not (Doyle and Herbinger 1994, Dupont-Nivet et al. 2002), although reproductive complications were not modeled in these studies. If strict walk-back selection is problematic, depending on the distribution of families in the upper tail of the phenotypic distribution, a stepwise approach of genotyping every second, third or fourth individual can be taken to obtain an adequate number of families at a reasonable cost with some sacrifice in selection intensity. Walk-back selection in its pure sense only applies to individually measured traits. Aggregate traits such as survival must be handled differently, through random sampling, which creates another set of complications to be conquered.

The aim of this dissertation is to determine the suitability of mixed family selection for breeding Pacific oysters using marker-based pedigree reconstruction and to test its efficacy relative to more traditional methods. This research addresses three important unanswered questions: 1) What is the most cost-efficient suite of genetic markers that can be used for reconstructing Pacific oyster pedigrees? 2) At what point in the life cycle can we mix families in equal numbers and expect them to still be equally represented when they are planted in the field? 3) What is the optimal strategy of selectively genotyping individuals to implement mixed-family selection and does it compare favorably with traditional separate family selection?

An overall goal of the project is to improve the efficiency of shellfish breeding in terms of minimizing the growing space and labor required, and determine the applicability of mixedfamily selection according to the two traits

survival and individual weight, the components of yield. When mixing families together rather than keeping them separate, the microenvironment of all families would be the same, theoretically reducing the level of replication and blocking required to account for environmental variation, and as a result, the number of growing bags needed could potentially be decreased as much as ten or twenty-fold, depending on the trait under selection. Our overall goal is pursued through a series of four objectives. Objective 1 is to develop a workable strategy to quickly identify the most efficient set of molecular markers that will accurately determine the parentage of mixed oyster samples from any breeding population. Objective 2 is to determine how early in their life-cycle different families of oysters can be mixed without subsequent distortion of their representation before deployment in the field. Earlier mixing saves hatchery space and effort, homogenizes microenvironmental effects earlier, and makes it more feasible to implement this approach in a commercial context. Objective 3 is to identify the most cost-effective strategy of selective genotyping and to test the mixed-family approach side-by-side with the current method of separate-family breeding. Finally, Objective 4 is to apply the mixed method to estimate the heritability of shell shape, an economically important characteristic of Pacific oysters for the halfshell market.

CHAPTER 2. P-LOCI: A COMPUTER PROGRAM FOR CHOOSING THE MOST EFFICIENT SET OF LOCI FOR PARENTAGE ASSIGNMENT

P-LOCI: a computer program for choosing the most efficient set of loci for parentage assignment

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Abstract

Determining how many and which codominant marker loci are required for accurate parentage assignment is not straightforward because levels of marker polymorphism, linkage, allelic distributions among potential parents and other factors produce differences in the discriminatory power of individual markers and sets of markers. P-LOCI software identifies the most efficient set of codominant markers for assigning parentage at a user-defined level of success, using either simulated or actual offspring genotypes of known parentage. Simulations can incorporate linkage among markers, mating design, and frequencies of null alleles and/or genotyping errors. P-LOCI is available for Windows systems at http://marineresearch.oregonstate.edu/genetics/PLOCI.htm

Program description

Parentage assignment using codominant molecular markers has become increasingly important for quantitative genetics, animal breeding, molecular ecology and evolutionary biology (Jones and Ardren 2003, Vignal et al. 2002, Anderson and Garza 2006). Determining the most efficient set of marker loci to use for a particular set of parents can save considerable time, effort, and funds. The minimum number of loci necessary to accurately assign parentage depends on a number of factors that affect their informativeness, including allelic richness and diversity, linkage disequilibrium (LD) among marker loci due to physical linkage and other sources, number of parental pairs, mating design, frequency of null alleles and genotyping errors, and unequal numbers of offspring per family (Anderson and Garza 2006, Bernatchez and Duchesne 2000, Dakin and Avise

2004, Jones and Ardren 2003, Kalinowski et al. 2007, Kalinowski and Taper 2006). Few currently available parentage software packages have multilocus predictive capabilities, and they do not incorporate many of these important factors (Jones and Ardren 2003, Taggart 2007). Most researchers and all currently available parentage software assume markers are not linked, even although physically linked markers carry redundant information and are thus less informative in combination than expected from single locus characteristics. P-LOCI is the only program that uses linkage information together with variable locus-specific frequencies of null alleles and genotyping errors in the simulation of offspring genotypes with variable number of offspring per family to determine the minimum set of loci for assigning parentage. Additionally, because the best combination of loci can vary among populations, marker informativeness must be re-evaluated for each study population, creating the need for a quick and easy to use software tool. We created P-LOCI to increase the efficiency of parentage assignment by quickly identifying the best available set of codominant molecular markers for parentage assignment in a specific population. Figure 2.A.1 shows the P-LOCI interface with an explanation of the controls.

P-LOCI identifies the smallest suite of codominant loci required to assign diploid offspring to their parents at a user-defined level of success through an iterative procedure. In either simulation or real progeny mode, the user provides a parental file consisting of the candidate parents' multi-locus marker genotypes at all loci to be evaluated and a mating design file specifying how the parents are paired. When the mating structure is not known, the user submits an all-

combinations mating file. P-LOCI simulates offspring genotypes using those files and optional linkage and error information, and then attempts to assign them to their parents based using an exclusion algorithm. The accuracy of these assignments is evaluated against the known pedigrees of simulated or actual progeny. P-LOCI was created primarily for use with microsatellite data, but works with any codominant genetic markers. Figure 2.A.2 is a conceptual model of P-LOCI, showing information flow.

P-LOCI simulates biologically realistic offspring genotypes through a computationally intensive but genetically realistic "brute force" procedure by first building virtual gametic haplotypes from each parent. For each virtual offspring, the program first randomly chooses one allele from the current parent at the first locus in each linkage group and then "walks" along the virtual parental meiotic chromatid. Cross- over probabilities between adjacent markers are determined by recombination fractions calculated from linkage map distances. If a linkage map is not provided, the program assumes independent marker segregation and assembles each haplotype choosing each allele at each locus with equal probability. The two haplotypes are then combined into a diploid offspring according to the mating design.

P-LOCI accommodates different male and female maps in either Kosambi or Haldane distances (Liu 1997, Lynch and Walsh 1998). Linkage phase among marker alleles in specific parents is assumed as their order of entry in the parental genotype file. If the user knows the phase, they can enter it as such, although the true phase is usually unknown, and therefore arbitrarily represented in genotype

data. The user can also vary the number of offspring produced per family. This may be desirable if some families are expected to be over-represented in the offspring pool, or to model variability in the best marker set, due to variance in relative contribution of specific parents to the offspring population.

P-LOCI can realistically incorporate two types of error when simulating offspring genotypes: segregating null alleles and random genotyping errors. P-LOCI optionally introduces null alleles at user-specified frequencies and creates a modified parental genotype file in which a proportion of the homozygous parents at each locus are re-coded as heterozygotes with an undetectable null allele. The simulated offspring that inherit these null alleles are treated by the assignment algorithm as homozygotes for the detectable allele. Null allele frequencies and genotyping error rates must be estimated by the user *a priori* using other available software (e.g. Kalinowski et al. 2007, Van Oosterhout et al. 2004). The user can also designate individual parents *a priori* as null homozygotes.

P-LOCI optionally incorporates microsatellite marker typing errors by randomly adding or subtracting a user-defined number of base pairs to the offspring alleles, producing mismatches and potential misassignments that realistically compromise the discriminatory value of error-prone loci. To mitigate errors in real or simulated data sets that prevent assignment via exclusion, the user can enter a maximum number of loci at which offspring are allowed to mismatch potential parents and still be assigned to them. The conservative user can also have P-LOCI determine a marker set with one more locus than is needed to reach the assignment success criterion.

If information regarding the rates of null alleles, typing errors or linkage relationships among markers is not available, the user may wish to genotype a small number of offspring of known parentage from all crosses (e.g. offspring of controlled crosses or observed matings) at all loci in order to produce an input file containing actual offspring genotypes rather than simulated ones. Actual offspring genotypes will inherently exhibit the effects of the aforementioned complicating factors.

After P-LOCI either produces the simulated offspring file or is provided with actual offspring genotypes of known parentage, the user initiates the marker evaluation algorithm, and P-LOCI first assigns all offspring using each marker individually by checking each offspring for Mendelian compatibility with each parental pair in the mating file. Assignments are successful only when a single compatible parental pair is identified. If more than one compatible pair is found, or if an offspring is misassigned when checked against its known parentage information, that individual assignment is unsuccessful. This information is used to rank individual loci by their assignment success rate. The software subsequently examines all possible marker pairs, triplets, etc., and stops when it reaches the user-provided level of assignment success. The program then produces a report that includes the ranks of individual loci and their assignment scores, followed by the best pair, triplet, and so on. P-LOCI can automatically produce and analyze multiple sets of simulated offspring and produce a summary report that includes the average rankings of individual loci among runs and how often a particular locus appeared in the best marker set. After using P-LOCI to determine the best

set of loci, the user can assign actual progeny to their parents using a variety of methods and software, of which Jones and Ardren (2003) provide a thorough review.

We tested P-LOCI with actual and simulated microsatellite and SNP data, varying levels of polymorphism, distribution of alleles among parents, number of parents, mating design complexity, degree of linkage among markers, and locus-specific frequencies of null alleles and genotyping errors. P-LOCI reported increasing assignment success with increasing allelic richness and more heterogeneous allelic distributions among potential parents. Assignment success decreased with increasing number of potential parents, increasing complexity of the mating design, and higher frequencies of null alleles and genotyping errors. In general, unlinked markers provided a higher level of assignment success than linked ones, all other factors being equal. P-LOCI also chose different marker sets in different parental populations of Pacific oysters, *Crassostrea gigas* (Matson and Camara, Langdon and Evans, unpublished data), using genotype data from breeding experiments, microsatellite markers, and microsatellite linkage map information (Hubert and Hedgecock 2004). We used an early version of P-LOCI to determine the best available suite of microsatellites (Li et al. 1998, Magoulas et al. 1998, McGoldrick et al. 2000) for assigning 1200 offspring to 20 pairs of parents, and performed parentage analysis with a 98.5% success rate using PAPA software (Duchesne et. al. 2002) and four loci.

Single locus ranks within populations were similar to those obtained from ranking loci by Shannon Diversity Index computed with Microsatellite Analyser

(Dieringer and Schlotterer 2003). However, we found that the best suite of loci often consists not of only the top-ranked individual loci, but rather a mixture of top- and middle-ranked markers. This is most likely due to random allelic associations among loci, and LD in the parental population that make the information carried by some marker sets redundant and others complementary.

Our preliminary results have important implications. The top ranked individual loci do not necessarily constitute the smallest group of loci for assignment, and that group is not necessarily the best for all populations, making P-LOCI an important tool for efficient parentage analysis.

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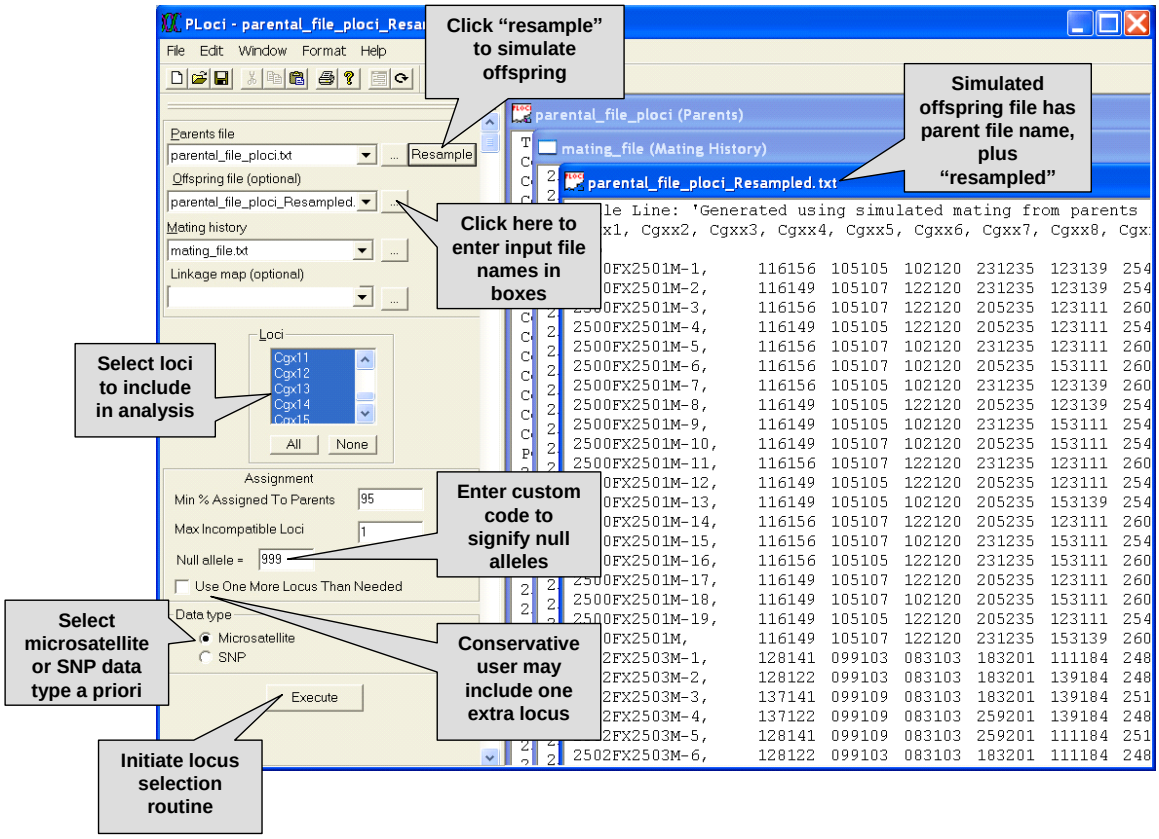

Figure 2.A.1. Screenshot of P-LOCI software interface, showing where to enter input files and other important operating information.

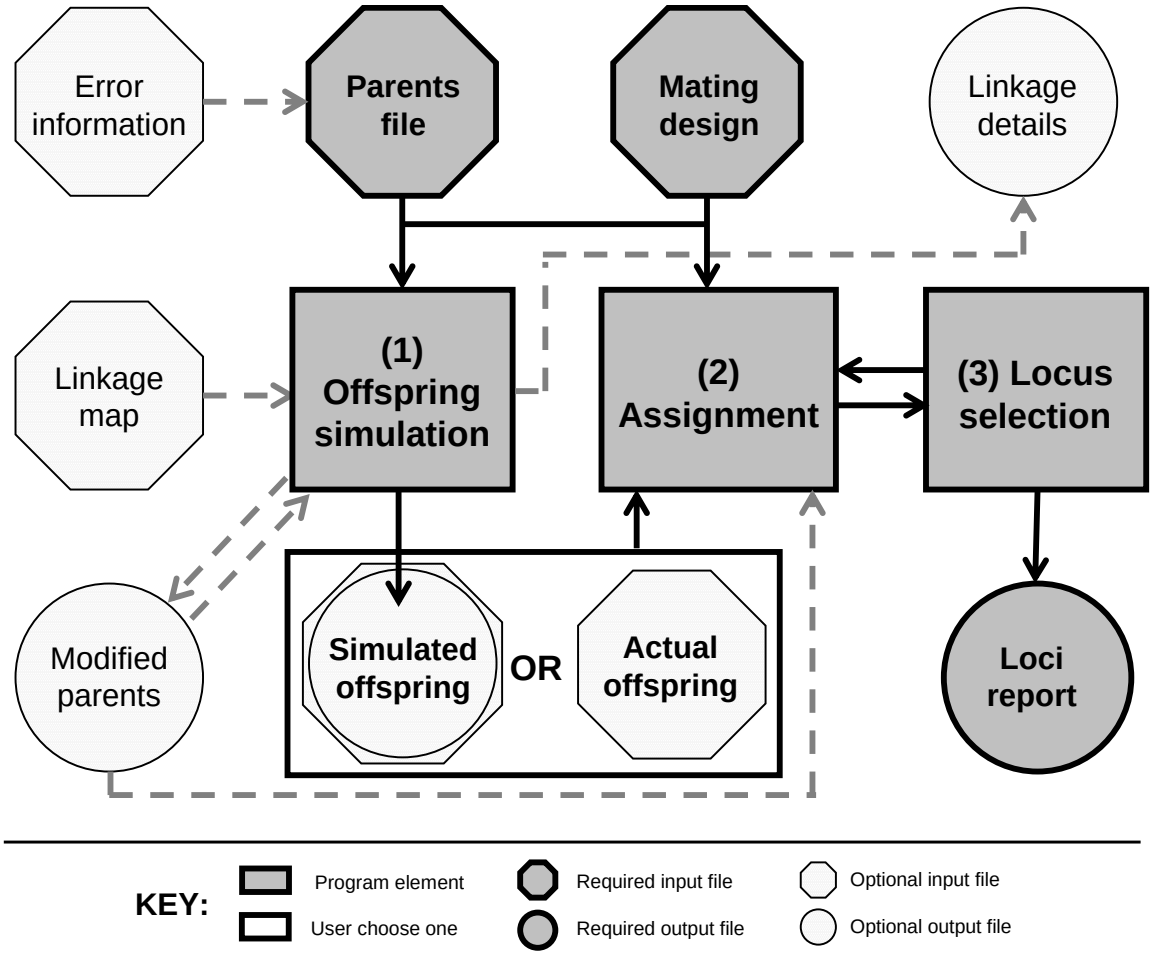

Figure 2.A.2. Conceptual model of P-LOCI showing information flow, signified by arrow direction. Program operation is depicted as follows: 1) P-LOCI simulates offspring (or they are provided by the user); 2) Those offspring are assigned to candidate parental pairs, as denoted in the mating design; 3) The locus selection routine ranks locus sets by assignment success, and either accepts the locus set as fulfilling the assignment success criterion, or reinitiates the assignment routine to perform all possible combinations of the next level (e.g. from pairs to triplets), or the current locus combination fulfills the criterion, the program stops and reports.

CHAPTER 3. MIXED FAMILY SELECTION IN THE PACIFIC OYSTER: DETERMINATION OF OPTIMUM LIFE-STAGE FOR MIXING

Abstract

Communally rearing oysters of different pedigrees for subsequent evaluation and selective breeding communally offers numerous potential advantages over the common method of among-family selection with families reared separately. Although it is economically desirable to mix individuals of different families together as early as possible, the oyster's complex life cycle makes it critical to identify the stage of development when families can be mixed and yet maintain approximately equal representation of each family at planting size for field trials. To address this, we conducted an experiment in which we mixed together equal numbers of offspring from 20 different full-sib oyster families at three stages of development. We reared them for 48 days post-fertilization, until they reached a size typical of planting for field trials. After genotyping and assigning offspring to their parental pairs, we found that variance in family representation within a mixture increased dramatically with the time elapsed since mixing. We also found that family representation within mixes was no longer equal at 48 days post-fertilization (planting size) in groups that were mixed at 24 hours (straight-hinge larvae) and 13 days (pediveliger larvae), while groups that were mixed at 27 days (post-larvae) remained uniformly mixed. Our results indicate that given any additional variability due to periodic unexpected nursery mortalities, the most prudent choice of time to mix oyster families is at planting size, for selection on field traits. Mixing at this stage also maximizes initial information and minimizes genotyping costs.

Introduction

The Pacific oyster (*Crassostrea gigas*) is the most widely cultured mollusk and its worldwide production currently exceeds that of any other marine or freshwater organism (FAO 2005). In order to increase production efficiency and product value, selective breeding programs have been established in many countries including the USA, France, Australia, and New Zealand. Pacific oyster breeding is complicated by this species' high fecundity, high variance in reproductive success and high genetic load (Launey and Hedgecock 2001), all of which can result in rapid loss of genetic variation unless a preventative strategy is followed (Evans et al. 2003, Newkirk 1978).

One such strategy used in oyster selective breeding is among-family selection with families reared separately. Although it can be used effectively to control inbreeding and requires low genotyping effort (parentage confirmation of broodstock), this separate-family approach has some statistical, economic and practical disadvantages. First, among-family genetic effects are partially confounded by environment and family-specific density effects. While large-scale environmental variation can be accounted for by blocked and replicated experimental designs, family-specific survival rates decrease the number of oysters within bags by different amounts, and this can influence growth rates in density-dependent fashion. Another disadvantage of the separate-family approach is that it requires the use of a research-scale hatchery and nursery with many, small, family-specific growing units. This necessitates substantial additional labor and special equipment for husbandry compared with a typical commercial hatchery and

nursery consisting of few very large units. The replication and blocking that is necessary for field trials to accommodate plot environmental variation also entails a large number of growing units, labor to maintain them, and large field plots. In addition, oysters bred using this method are reared only among their siblings, rather than among unrelated individuals, the way Pacific oysters are typically grown in the production environment on the West Coast of the United States. This is potentially problematic since the ultimate goal of breeding efforts is to change trait values in the production environment.

These difficulties could potentially be overcome by a mixed family selection (MFS) method. Using MFS, the breeder creates the same crosses they ordinarily would, but mixes them together at some point in the life cycle and later separates the families at harvest for measurement, evaluation and selection of replacements. In MFS all families experience the same environment among individuals from a variety of pedigrees. This prevents the confounding of genetic effects with microenvironmental effects (density within bag) and increases similarity between the breeding and commercial production environments (although random position within bags should affect both methods equally). MFS could also speed genetic progress in a breeding program by enabling the production of additional crosses. In the separate-family breeding method, the total number of families that can be produced for evaluation and selection is limited by the number of separate growing units that can be housed and maintained in breeding facilities. Rearing families mixed together enables the breeder to produce a much larger number of families in one or a few large units. Given a constant

amount of phenotypic variation and heritability, increasing the number of families would increase selection intensity, resulting in increased response to selection, from an among-family selection perspective.

Doyle and Herbinger (1994) introduced their version of mixed family selection to aquaculture, coining the term "walk-back selection". Walk-back selection entails harvesting a mixed-family group of organisms, measuring them for some trait, ranking them, and then "walking back" the phenotypic distribution until replacements from enough families have been selected to produce another generation. The method focuses on individual selection, while paying attention to family identity to avoid inbreeding. It could also be used to perform among-family selection or combination selection. Since 1994, walk-back selection has been explored with several aquaculture species including Atlantic salmon (Herbinger et. al. 1999), Norway lobster (Streiff et. al. 2004), African catfish (Volckaert et. al. 1999) and European lobster (Jorstad et. al. 2005). MFS is very amenable, but not limited to walk-back selection, and any variation of the mixed family method relies on the ability to identify individuals to family at harvest.

Simply tagging each oyster before mixing families together is a logical approach, however, tagging oysters at early stages in the life cycle is not currently feasible on the scale necessary for MFS. There is currently no method known for marking oyster larvae for scores or hundreds of different families. Juvenile oysters show poor retention rates for glued-on tags, and oysters must be already adult before it is possible to drill a small hole through the hinge to attach a tag using monofilament line. Injected microwire tags may have potential for oysters of

planting size or larger and PIT tags, which are much larger, for yearling oysters. Coded wire and PIT tags have been successfully applied and retained at high rates in an external ligament the brackish water clam, (Lim 1999) and internally to small adult freshwater mussels (55mm) respectively (Kurth 2007). However, the aforementioned methods are all labor intensive for large numbers (i.e. thousands) of small bivalves (ones lacking external ligaments), and they do not enable tagging of oysters as larvae or post-larvae, when they are less than two millimeters in length. An acceptable tag for oyster MFS would need to be retained for at least two years, through multiple life stages, and a change in mass of hundreds of times while living in a rough intertidal or subtidal marine environment. Currently, only molecular markers fulfill these requirements.

We chose microsatellite markers for our study because of their high degree of polymorphism and power for parentage assignment. Also, there were more than one hundred of them available in this species at the time of the study (Li et al. 1998, Magoulas et al. 1998, McGoldrick et al. 2000, Sekino et al. 2003) and no SNPs which were suitable for parentage assignment.

Fundamentally, the MFS breeding cycle proceeds through the following steps; 1) production of offspring from different families, 2) mixing of equal numbers of individuals from each family into a pool, 3) rearing them together in the same environment(s), 4) harvesting the oysters after growth to a marketable size (which takes two years for *C. gigas* in the Pacific Northwest of the USA), 5) sampling oysters at harvest time, 6) assigning sampled individuals to family and 7) selecting replacements from those sampled, to produce another generation of

offspring.There are three critical points in this cycle that will have the largest influence on the method's efficiency: finding the optimum time to mix families (step 2), determining the sampling scheme to employ at harvest to efficiently collect and choose adequate replacements (step 5), and minimizing the costs of genotyping to separate the families in a mixed sample (step 6). Our research focuses on optimizing MFS for the Pacific oyster concentrating on these three points.

We addressed Step 6 by developing software to determine the optimum suite of codominant loci for assigning offspring back to their parents (Matson et al. 2008, Chapter 2), and addressed Step 5 in a subsequent field study (Chapter 4). Our objective in this study was to address Step 2, and empirically determine the most efficient stage of development to mix oyster families together for selection. The questions we asked were: 1) Is there a life-stage when we can mix together progeny so that family representation within each mixture is still equal when the oysters reach planting size, and 2) How does the variation in family representation at planting size change according to the stage of development at which families were mixed together?

Methods

Experimental design

We performed an experiment in a hatchery and a land-based nursery system in which we mixed together offspring from 20 different oyster families at three stages of development: straight-hinge larvae (also called D-larvae, 24 hours post-fertilization), pediveliger larvae (13 days post fertilization), and post-larvae

(very early juveniles, 27 days post fertilization). We replicated each mix four times, and reared the oysters mixed together until 48 days post-fertilization, whereupon they attained an average length of 8mm. At this point, we sampled 96 individuals from each of the twelve mixed groups, genotyped them, and assigned them to family with a 98% success rate using PAPA software (Duchesne et al. 2002), using a set of four microsatellite markers (Li et al. 2003, Magoulas et al. 1998, McGoldrick et al. 2000) that we chose with an early version of our P-LOCI software (Matson et al. 2008).

Choice of time points for mixing

We chose to evaluate these three time points for mixing because of their significance in the oyster culture cycle, and because each stage is a landmark for oyster survival. We waited until the straight-hinge stage, at 24 hours, to avoid the potentially extreme amount of variability due to fertilization success and sperm viability. At 24 hours post-fertilization, the larvae have shells, measure approximately 60 microns, have begun feeding, and are much more durable than previous stages, which is desirable for sieving and counting them. By the pediveliger stage at 13 days, the larvae have changed dramatically. Having increased in length by five times, they have developed an eyespot and a chemosensory adhesive foot with which to judge suitable substrate and attach to it. This stage marks the end of the larval phase of life and the now "competent" larvae are ready to metamorphose into sessile juveniles. Their large size of approximately 300 microns makes them easy to handle and count, and being at the end of the larval phase makes this another logical, convenient point for the breeder

to mix individuals from different families. At our third mixing point, approximately two weeks after metamorphosis, the oysters can be called post-larvae, as they are sessile and have well-developed shells. They have grown larger than 1mm, and are resilient enough to tolerate minor environmental insults, such as aggressive sieving, counting, and rinsing with fresh water to discourage pathogens.

Oyster propagation and husbandry

We conditioned broodstock oysters in 18° C sand-filtered seawater from the Yaquina Bay at the Hatfield Marine Science Center in Newport, Oregon and fed them a mixture of *Isocrysis galbana* (TISO), *Chaetoceros calcitrans* (CC), *Chaetoceros mulleri* (Chagra), and *Tetraselmis striata* (Tet) at concentrations of 50,000–80,000 cells mL^{-1} for several weeks until they were in fully reproductive condition (Robinson 1992a, 1992b). We strip-spawned those broodstock and made twenty full-sib pair-matings; we fertilized eggs and incubated crosses separately at 25°C for 24 hours. After reaching the straight-hinge stage, we made three counts of normally developing straight-hinge larvae from each cross, and stocked them in each experimental bucket (volumetrically) to achieve an initial density of three larvae ml^{-1} in 30 liters of seawater. Replicate larval mixtures of different families were produced at equal density. Seawater for larval cultures was pumped from the Yaquina Bay at high tide and passed through sand filters, followed by a 20 µm cartridge filter and given a daily addition of calcium montmorillonite according to Matson et al. (2006). Larval cultures were reared at 25°C in 30L tanks with aeration in a temperature-controlled room (Langdon et al. 2003) and fed equal

rations by cell number of CC and TISO phytoplankton strains to slight excess daily according to a standard schedule from 30,000-80,000 cells ml^{-1}, depending on age (Breese and Malouf 1975). We changed water every other day. Larvae were retained on 40 µm sieves on days one and three, 80 µm sieves on days five and seven, 180 on days nine, 11, and 13. At day 13, pediveliger larvae retained on a 243 µm sieve were induced to metamorphose using an epinephrine solution at 2×10^{-4} M for 1 hour to induce metamorphosis (Coon et al.1986). Larvae were sampled at 10 days post-fertilization and preserved in 0.375 ml buffered 37% formaldehyde and 4 ml seawater for later measurement. Growth was measured as shell length (longest measurement) at age (10 days) using an ocular micrometer at 250X on a Nikon light and phase contrast microscope. Larval size distributions were negatively skewed, showing no truncation at the small end of the distribution, so sieving was unlikely to be a source of bias in family representation.

Successfully metamorphosed postlarvae from each tank were transferred to 15 cm diameter convertible upwelling/downwelling silos. Silos were held in a semi-recirculating system that received approximately 20 exchanges day^{-1} of sand-filtered seawater. They were configured to downwell for the first three weeks and then were changed to upwelling configuration as the oysters grew larger and heavier. The temperature of the system was held at 24° C until all the larvae had metamorphosed, and then it was decreased by 2° C per week until reaching ambient water temperature, which remained at 15° C (±2° C) for the remainder of the experiment. Nursery oysters were fed a TISO/CC/Chagra/Tet mixture at a final

concentration of approximately 50,000–80,000 cells ml^{-1} for approximately 20 hours per day.

We produced four replicate mixtures at each of three time points; 24 hours, 13 days, and 27 days post-fertilization. We created mixtures at equal densities within and among treatments and sieved them equally and conservatively, so as to retain as many live individuals as possible, while discarding dead ones in accordance with standard hygienic hatchery and nursery practices. Post-larvae were reared in downweller silos with 180 um sieves, and then transferred to 450 µm sieves and upwelled when individuals were large enough. Individual silo positions within each tank were rotated daily. Replicates within treatments were transferred at the same time.

Family assignment

We sampled adductor muscle from parental oysters and fixed it in 4 ml tubes of 95% ethanol. We crushed whole progeny 8 mm spat, one into each 1.5 ml centrifuge tube, and fixed them in 95% for later extraction using Quiagen DNEasy kits. We diluted extracted DNA 4X in 1X TE and used 1 µl of this working stock for PCR. We genotyped the parents at 16 microsatellite loci (Li et al. 1998, Magoulas et al. 1998, and McGoldrick et al. 2000) to enable choice of the minimum suite of markers necessary for assignment of the progeny using an early version of P-LOCI software (Matson et al. 2008). We genotyped progeny at four loci and assigned to pairs of parents using PAPA software (Duchesne 2002). We optimized microsatellite loci and performed PCR on an MJ Research PTC-225

Peltier Thermocycler and ran PCR products on an ABI 3730XL genotyper/sequencer.

Statistical analyses

We tested among-family frequency distributions of the three different treatments against the null hypothesis of a uniform distribution using

Fisher's exact randomization test with Monte-Carlo estimation of p-valures (McDonald 2008, Sokal and Rohlf 1996), (also in SAS). It compares a Chi-square statistic with the value of that statistic for other random rearrangements of the data. The test pooled replicates within each treatment, and thus sacrificed consideration of variation within treatment, for a very robust procedure.

We used a Conover squared ranks test for homogeneity of variances among treatments (i.e. mixed at straight-hinge, pediveliger, or post-larvae stage) to test whether the amount of variance within treatment differed among the three treatments, using SAS software. P-values were estimated for this procedure using a Fisher z-transformation

Results

Goodness of fit

The output from the Monte-Carlo estimate of Fisher's exact test using SAS PROC FREQ includes Chi-square values, degrees of freedom, p-values for the standard Chi-square tests, as well as the Monte-Carlo estimated p-values and 99% confidence intervals for the p-values (Table 3.1). Under the exact test, SH and PV treatments both were significantly different from a uniform distribution

(p_{SH}=0.0000, p_{PV}=0.0018, Table 3.1, Figure 3.1), while PL was not significantly different from uniform (p_{PL}=0.9520). Asymptotic Chi-square values and corresponding p-values were as follows: χ^2_{SH}=114.4648, p<0.0001; χ^2_{PV}=42.3887, p=0.0016, χ^2_{PL}=10.0765, p=0.9510, (Table 3.1).

Variance in family representation by treatment

Variance in family representation within mixtures increased proportionately with the amount of time that families spent mixed together (Figure 3.2). The post-larval treatment, which was mixed at 27 days and sampled at 48 days (21 day mixed duration) had the lowest among-family variance from the pooled replicate samples with a coefficient of variation (CV) of 41 %, while the pediveliger treatment, mixed at 13 days and sampled at 48 days (35 day mixed duration) had higher among-family variation with a CV of 58 %. The among-family variation in the straight-hinge treatment, mixed at one day and sampled at 48 days (47 days mixed duration) was the highest with a CV of 70 %.

Few families (1, 6, 15, 18 and 20) maintained similar representation irrespective of mixing time, while most departed further from initial proportion with increasing time reared mixed together (Figure 3.3). A Conover squared ranks test of homogeneity of variances showed that variance differed significantly among treatments (*p*=0.0063).

Discussion

Our experiment clearly demonstrates the dramatic effect of the time of mixing upon family representation within mixtures at planting, which has major

implications for utilizing MFS. The time to mix families depends upon the trait under selection, and whether it is measured aggregately or individually. We found that although the potential for savings in husbandry equipment and labor are highest for mixing at the earliest possible stage, the high variability in family representation associated with early life stages is prohibitive for aggregate field traits, such as survival.

Table 3.2 presents seven different MFS scenarios of mixing times and traits that include three components of effort. Each row indicates a different combination of trait type and mixing time. Within each combination, effort is assigned dichotomously as either low = “L” or high = “H”, for each category (Hatchery, Nursery, and Genotyping). These scores can be considered as rough proxies for cost.

Mixing at planting size would be the most prudent choice for selection on field traits, but we will consider it as one of three main options for mixing times: first, mixing as larvae (either SH or PV); second, as post-larvae (PL); or third, at planting size. Mixing as SH larvae would enable one to eliminate family-specific hatchery tanks and nursery equipment all-together; families would be fertilized in separate buckets, and then mixed 24 hours after fertilization and reared in one or a few large hatchery tanks, followed by a few, large nursery upweller silos, maximizing benefit from economy of scale (Table 3.2, option A or B). Mixing at PV would offer less opportunity for savings, because of the need for rearing families in separate tanks until metamorphosis (Table 3.2, option C or D). Mixing at either SH or PV restricts the ability to estimate family representation and initial

size to only the case of genotyping a large sample of juveniles at planting, which would likely be cost-prohibitive. Alternatively, one could ignore early variability and select only on individual traits, or on whole-life performance, across all life stages. The latter strategy has two flaws; one is that the high level of hatchery and nursery variability in family representation, which we observed in this study, would confound field survival. Our results also demonstrate that mixing families as larvae would lead to the loss and/or rarity of some families (Figure 3.2). This would reduce confidence in the estimation of means for rare families, and bias within and among-family variance (and selection intensity) estimates.

Mixing as post-larvae (PL) offers little opportunity for savings over mixing at planting size. The breeder would need to rear families in separate containers through the larval stage, and part of the nursery stage (Table 3.2, option E). Although the PL treatment showed no significant difference from equal family representation, there is a significant risk of variable family representation if mortalities occur in the nursery. Although this experiment showed optimal growth and survival, with families synchronously reaching metamorphosis by day 13, and planting size by 48 days after fertilization with negligible mortalities, it was a best-case scenario. Experienced culturists will attest that significant mortalities will periodically occur at any stage in production, including between PL and planting size. Thus, it is important to consider that post-larval mortalities would likely bring about significant variability in family representation. Also, although this experiment was thoroughly internally replicated, variation in survival and growth can be expected among hatcheries and among seasons.

Planting size is arguably the most prudent time to mix families (Table 3.2, option F). It offers many distinct advantages. First, this option avoids the variability of early life stages. It also enables the measurement of initial size and family representation, which maximizes the accuracy of aggregate and initial trait estimation. Because of this, the breeder needs only to genotype at harvest, which saves sizeable expenditures. It is also safer than mixing as PL if there are nursery mortalities.

Twenty full-sib families were enough for us to answer the key questions in this study with sufficient statistical power. However, breeding programs using among-family selection will likely produce more than 20 crosses. For example, the Molluscan Broodstock Program (MBP), a Pacific oyster breeding program at Oregon State University, currently produces 50 to 60 families per cohort. According to simulations using our P-LOCI software (Matson et al. 2008), we estimated that one can assign parentage of 50 to 60 full-sib crosses using four or five highly polymorphic microsatellite loci with nearly 100% success, although the most efficient suite of loci varies by population, and that number would not apply to all populations or mating designs. The number of loci necessary to assign parentage at a given level of success depends on the number of parents used, complexity of the mating design, and the relatedness among the parents, as well as the allelic diversity of each locus and the linkage disequilibrium within a given set of loci. The sample size needed to estimate family representation at a given level of confidence increases with the number of families in the population. These are important issues for breeders to consider when planning MFS.

Conclusions

Our experiment has clearly shown that variance in family representation within mixtures increased with the amount of time elapsed since mixing occurred. Family representation within mixes was no longer equal at 48 days post-fertilization (planting size) in groups that were mixed at 24 hours (straight-hinge larvae) and 13 days (pediveliger larvae), while groups that were mixed at 27 days (post-larvae) remained uniformly mixed. While post-larval mortalities in this experiment were negligible, periodic mortalities should be expected at any stage of production, and could easily lead to high variability in family representation at the PL stage as well. For these reasons, and others mentioned earlier, planting size is the most prudent time to mix families for MFS. Mixing families at stages earlier than this for selection on field traits would require pre-planting genotyping for estimation of initial family representation or other special considerations.

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Table 3.1. Goodness of fit test results; Fisher's Exact Test with Monte-Carlo estimated p-values.

STRAIGHT-HINGE			**PEDIVELIGER**			**POST-LARVAE**		
Chi-Square Test for Specified Proportions			Chi-Square Test for Specified Proportions			Chi-Square Test for Specified Proportions		
Chi-Square	114.4868		Chi-Square	42.3887		Chi-Square	10.0765	
DF	19		DF	19		DF	19	
Asymptotic Pr > ChiSq	<.0001	s	Asymptotic Pr > ChiSq	0.0016	s	Asymptotic Pr > ChiSq	0.9510	ns
Monte Carlo Estimate for the Exact Test			Monte Carlo Estimate for the Exact Test			Monte Carlo Estimate for the Exact Test		
Pr>= ChiSq	0.0000	s	Pr>= ChiSq	0.0018	s	Pr>= ChiSq	0.9520	ns
99% Lower Conf Limit	0.0000		99% Lower Conf Limit	0.0014		99% Lower Conf Limit	0.9503	
99% Upper Conf Limit	0.0000		99% Upper Conf Limit	0.0021		99% Upper Conf Limit	0.9538	
Number of Samples	100000		Number of Samples	100000		Number of Samples	100000	
Initial Seed	632857001		Initial Seed	557040000		Initial Seed	775043001	
Sample Size =	378		Sample Size =	373		Sample Size =	379	

Table 3.2. Comparison of effort by category through planting for hatchery, nursery and genotyping needs of different mixing times and traits using MFS, ranked by dichotomous relative effort scores within each category and summed across categories (right column). Each row indicates a different strategy comprised of a combination of trait type and mixing time. IND = individual, AGG = aggregate, SH = straight-hinge, PV = pediveliger, PL = post-larvae.

			Hatchery needs		Nursery needs		Genotyping needs		
Rank	Mix time	Trait	Equipment	Effort	Equipment	Effort	Individuals	Effort	Score
1	SH	IND	One or few large tanks	low	Few large silos	low	parents	low	LLL
2	SH	AGG	One or few large tanks	low	Few large silos	low	parents, pre-plant	high	LLH
2	PV	IND	Many small tanks	high	Few large silos	low	parents	low	HLL
3	PV	AGG	Many small tanks	high	Few large silos	low	parents, pre-plant	high	HLH
3	PL	either	Many small tanks	high	Many small silos (2 weeks)	high	parents	low	HHL
3	JUV	either	Many small tanks	high	Many small silos (4 weeks)	high	parents	low	HHL
3	Never	either	Many small tanks	high	Many small silos (4 weeks)	high	parents	low	HHL

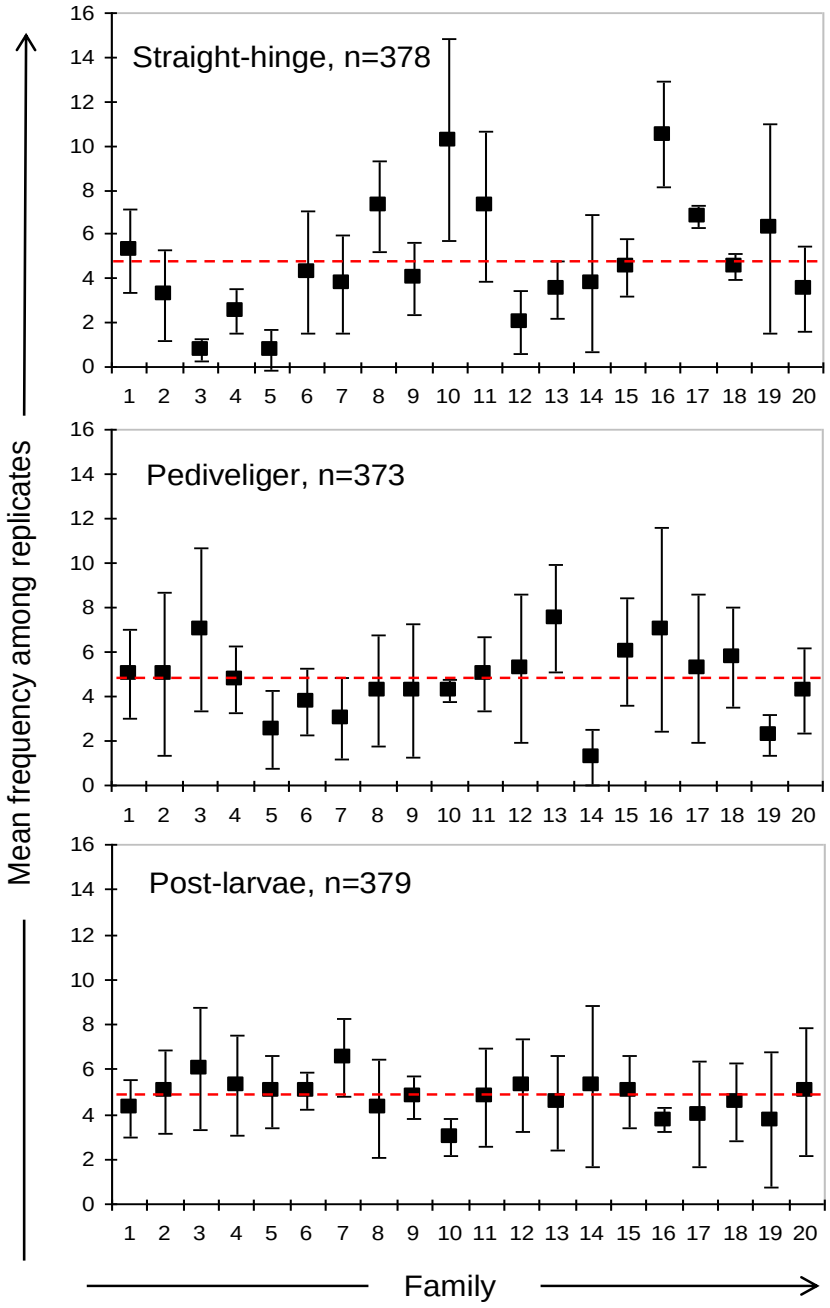

Figure 3.1. Mean family frequency in samples taken at 48 days post-fertilization for three different mixing times (± std. dev., n=four). Family number is on the x-axis, and frequency of offspring from each family is on the y-axis. The dotted line represents the null hypothesis of a uniform mixture (equal family representation).

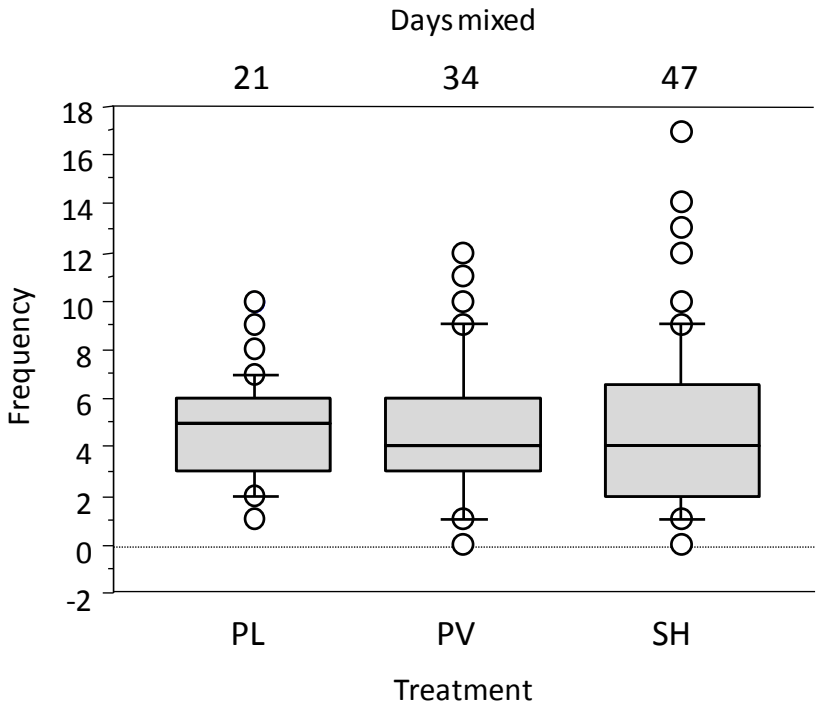

Figure 3.2. Boxplot, showing variation in family representation at the end of the experiment (frequency). Treatment is listed on the bottom x-axis; PL=post-larvae, PV=pediveliger larvae and SH=straight=hinge larvae. The number of days that each treatment spent mixed is listed on the top x-axis.

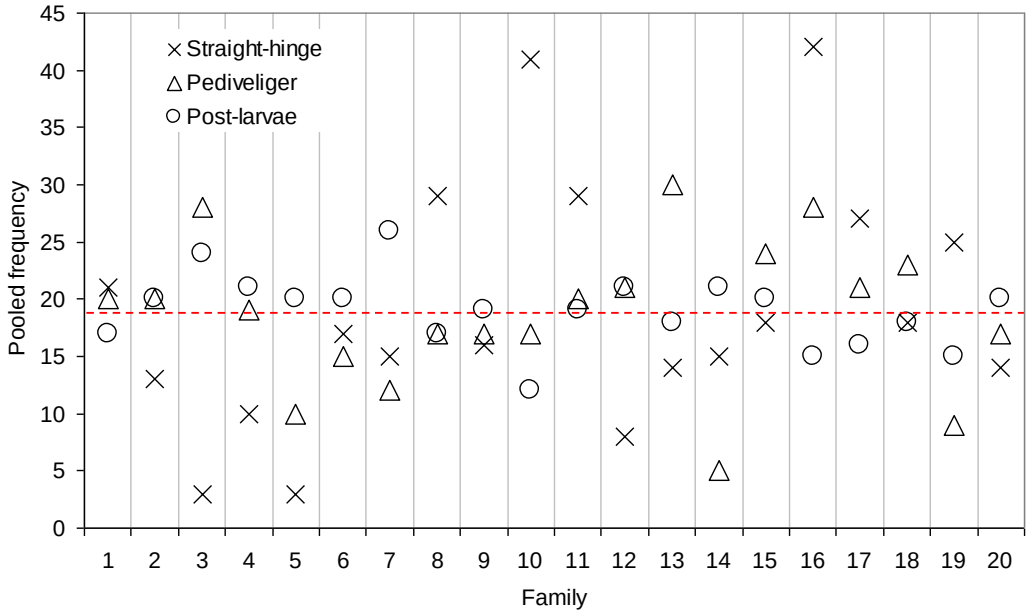

Figure 3.3. Variation in family representation among the three mixing times, shown as total frequencies (pooled replicates). The straight-hinge treatment is represented by Xs, pediveliger by triangles, and post-larvae by circles. The red, dotted horizontal line represents the expected frequency for a uniform mixture.

CHAPTER 4. FIELD EVALUATION OF MIXED VERSUS SEPARATE FAMILY REARING FOR SELECTIVE BREEDING IN THE PACIFIC OYSTER

Abstract

Pacific oysters are a highly economically important species with a global distribution, and are selectively bred in many countries. Selective breeding is typically done with families reared separately. This has some economic and experimental disadvantages which could potentially be remedied using mixed family rearing. Mixed and separate family rearing for selective breeding were compared empirically at two sites in the Yaquina Bay, Oregon, on the West coast of the USA, using 48 full-sib pedigreed oyster families in conjunction with the Molluscan Broodstock Program, of Oregon State University. Overall, rearing oysters of different families mixed together yielded very similar results to rearing them separately when comparing family mean individual weight and survival. This demonstrates it is unlikely that intraspecific associative effects are of great importance in the Pacific oyster at this range of stocking density. Differences in family-based performance values were likely due to sample size differences and high within-plot environmental heterogeneity at the intertidal test-site. Ranking of families based on walk-back selection for individual weight was very similar to ranking based on family mean individual weight. The mixed method was well-suited for selection by individual traits, but would incur high laboratory costs in order to estimate survival with acceptable levels of precision and accuracy.

Introduction

Pacific oysters have the highest global production of any aquaculture species, with 13 million tons produced in 2007 for a value of 12.8 billion US dollars (FAO 2010). This valuable species is selectively bred in many countries around the world, including France, Australia, New Zealand, Japan, and the United States. Its life history characteristics and genetics make it amenable both to aquaculture and to selective breeding. Some of those same characteristics, namely high genetic load (Launey and Hedgecock 2001) and high fecundity, also make it both sensitive to inbreeding, and particularly easy to inbreed unless measures are taken to avoid it (Newkirk 1978, Evans et al. 2003). Currently, selective breeding on the West coast of the United States is typically carried out with families reared in separate, marked units throughout the life cycle, and bred utilizing among-family or combination selection. This approach is effective at creating additive genetic change while controlling inbreeding (Langdon et. al. 2001), yet there are some aspects of this method that could be improved upon.

Some potential drawbacks to separate family rearing for selective breeding include that family-specific survival can unknowingly be confounded with growth effects through density-dependent growth, producing common environmental effects that reduce the accuracy of predicted breeding values. Also, family-specific mortality can potentially be exaggerated due to the presence of moribund oysters in the bags of those families with low survival, also reducing accuracy. Large amounts of labor, growing area and culture materials are needed to support the large experimental plots and large numbers of animals necessary to evaluate traits

under selection, and specialized equipment with large numbers of small growing units are needed to keep families separate during production before planting.

An alternative to breeding oysters with families reared in separate units is to mix different genotypes together for rearing, and separate them for evaluation and breeding. Individual tagging is not feasible in this species using current technology, due to tag shedding (C. Brooks, pers. Com.), the sheer numbers of individuals produced, and their small size at early life history stages. Genotyping using microsatellite markers and parentage assignment using computer software is currently the most feasible method. Rearing different pedigrees mixed together in the same growing unit removes potential common environmental effects by exposing all genotypes to all microenvironments equally. This approach could also potentially reduce the amount of blocking and replication needed to account for environmental variation, and in turn, reduce the size of field rearing areas and the amount of rearing equipment and labor needed. However, mixed animals need to be genotyped for identification, which adds significant cost, and it is also uncertain whether or not traits will be expressed similarly in the separate and mixed genotype environments. Additionally, all traits are not evaluated in the same way, and a method that works for one trait may not work for all.

The objectives of this study were to evaluate the performance of pedigreed Pacific oysters when either mixed or maintained separately in field trials as part of a selection program to improve survival and final weights at harvest. The comparison also included an evaluation of the practicality and economic costs of the two approaches. Results from this study will inform breeders of the relative

strengths and weaknesses of Mixed Family Selection (MFS) versus rearing families separately as part of a selective breeding program for Pacific oysters.

Methods

In the study, 48 full-sib oyster families were reared for two years in the same two experimental sites using both separate and mixed methods. At harvest, offspring were assigned to their parents and evaluated for survival and individual weight at harvest. Finally, the results from the two methods were compared.

Pedigreed families were produced as part of cohort 18 of the Molluscan Broodstock Program (MBP). MBP is a Pacific oyster breeding and research program, operated through Oregon State University at the Hatfield Marine Science Center (HMSC) in Newport, Oregon. Only full-sib families were used in this experiment both because of their availability (produced as part of this cohort), and to allow equal probability of assigning offspring to parental pairs. A mixture of full-sibs and half-sibs would have biased the probability of assignment success towards full-sibs. Families were reared in separate containers through the larval and juvenile phases.

Oyster propagation and husbandry

Broodstock oysters were conditioned in 18° C sand-filtered seawater from the Yaquina Bay at the HMSC and fed with a mixture of *Isocrysis galbana* (TISO), *Chaetoceros calcitrans* (CC), *Chaetoceros mulleri* (Chagra), and *Tetraselmis striata* (Tet) at concentrations of 50,000–80,000 cells mL^{-1} for several weeks, until they were in fully reproductive condition (Robinson 1992a, 1992b).

To produce full-sib pair-matings, we fertilized eggs and incubated crosses separately at 25°C for 24 hours. After organisms reached the straight-hinge stage, we made three counts of normal straight-hinge larvae from each cross, and stocked them volumetrically to achieve an initial density of three larvae per ml. Seawater for larval cultures was pumped from the Yaquina Bay at high tide and passed through sand filters followed by a 20 µm cartridge filter, and given a daily addition of calcium montmorillonite, according to Matson et al. (2006). Larval cultures were reared at 25°C in 30L tanks with aeration in a temperature-controlled room (Langdon et al. 2003) and fed daily with equal rations of CC and TISO phytoplankton strains, to slight excess according to a standard schedule from 30,000-80,000 cells ml^{-1}, depending on age (Breese and Malouf 1975). Water was changed three times per week. Larvae were retained on 40 µm sieves on days one and three, 80 µm sieves on days five and seven, 180 µm on days nine, 11, and 13. At day 13, pediveliger larvae, retained on a 243 µm sieve, were induced to metamorphose using an epinephrine solution at 2×10^{-4} M for 1 hour to induce metamorphosis (Coon et al.1986). Successfully metamorphosed postlarvae from each tank were transferred to 15 cm diameter convertible upwelling silos. Silos were held in a semi-recirculating system that received approximately 20 exchanges day^{-1} of 1µm-filtered seawater. The oysters were transferred to a larger upwelling system with a similar exchange rate as they grew to approximately 1.5mm in shell length. They remained in the large nursery system until approximately 4mm shell length, when they were moved to outdoor tanks at the ambient temperature of the Yaquina Bay. The temperature of the nursery system was held at 24° C until all the

larvae had metamorphosed, and then it was decreased by 2° C per week until reaching ambient water temperature, which remained at approximately 14° C for the remainder of the experiment. Nursery oysters were fed a TISO/CC/Chagra/Tet mixture at a final concentration of approximately 50,000–80,000 cells ml^{-1} approximately 20 hours per day.

It was empirically determined (see Chapter 3 of this dissertation) that mixing families as early veligers, pediveligers, or as post-larvae all produced significant departures from a uniform mixture by the time those oysters grew to planting size. Thus, for this study individuals from each family were mixed at planting size. Juveniles were randomly selected from separate family cultures for planting.

The oysters were planted at one intertidal and one subtidal site in the Yaquina Bay, at approximately 4mm shell length. One hundred growing units, each containing one individual from every family, were planted at each site and mixed randomly within the MBP (separate family) growing units. At the intertidal site, the growing units were 3/8 inch mesh ABS plastic growout bags, measuring 18 by 36 inches each. This site was divided into four blocks by tidal elevation; 25 bags were planted in each block, and were randomly interspersed among the separate-family (MBP) bags within each block. At the subtidal site, 100 growing units were divided among 13 lantern nets, each containing eight tiers (levels). The nets had 3/8 inch mesh. The 13 nets were randomly interspersed among the 52 separate-family nets. Nets at the subtidal site were divided into blocks by depth. Each block contained two tiers per net, and the tiers were evenly distributed

among blocks. The oysters were grown for two years. At one year, the oysters from both treatments at both sites were pulled up, removed from their bags, cleaned, scraped and put back. Bags at the intertidal site were turned over every three to six months, and nets at the subtidal site were washed with seawater using a firehose and high-volume pump every six months.

The separate-family treatment was harvested according to Langdon et al. (2003). Oysters from each bag were cleaned of fouling organisms and debris, after which the oysters from each bag were weighed as a group and counted. The entire harvest took approximately one week. The mixed family treatment was sampled at harvest; an equal number of bags within each block was randomly sampled (10 per block in the subtidal and 8 per block in the intertidal) until the number of oysters sampled approached 1200 at each of the two sites. The total number of oysters sampled was limited to approximately 2400 due to genotyping costs. Oysters were individually weighed live, and measured on a digital scallop measuring board (Scielex, Inc. Austrailia), after which, they were immediately shucked, sexed, and their stage of gonadal development was scored under a microscope. After this, a sample of mantle tissue was placed in 95% ethanol for later DNA extraction and genotyping.

Molecular methods and parentage assignment

DNA was extracted using a glass-fiber protocol according to Ivanova et al. (2008). Microsatellite markers were chosen by genotyping parents and analyzing those data using P-LOCI (Matson et al. 2008) to identify the most efficient set of markers for parentage assignment. P-LOCI identified Cg049, Cg108, and GL10

(Li et al. 2006, Magoulas 1998) as able to assign 95.5% of offspring back to their parental pairs in simulations. Parents and offspring were genotyped at those three microsatellite markers, plus an additional one to be conservative (Cg197, Li et al. 2006). The fourth marker, Cg197 added an additional 2.5% assignment success in simulations. Polymerase chain reactions (PCR) for the four markers were run separately using primers with four different dyes, but their products were coloaded on an ABI 3730XL fragment analyzer.

Parentage assignment was carried out using PAPA software (Duchesne et. al., 2002), with sex-differentiated, blocked parent files, using the three microsatellites Cg049, Cg108, and GL10. The settings used for genotyping error modeling included a global level of transmission error of 0.05 and a distribution of transmission error over alleles of 6, slightly more stringent than Taris et al. (2005) or Evans et al. (2009). The fourth marker, Cg197 was left out of the assignment analysis because it was unnecessary to meet the target level of assignment success, and it had higher than expected rates of large allele dropout and binning irregularity. The three loci enabled assignment with a success rate of better than 95%, as predicted by P-LOCI.

Data and analyses

Individual weight was measured directly for the mixed method; for the separate method, it was calculated by dividing the total raw bag weight by the number of individuals in the bag. Survival was calculated in the separate method as the number alive in a bag after two years, divided by the initial number in the bag at planting. Survival in the mixed method was calculated by dividing the

number of individuals present for a particular family within one block by the initial number of individuals planted for that block. Both measures of survival yielded proportions, which were then arcsine, square root transformed to adhere to normality and homogeneity of variance assumptions of the GLM (Sokal and Rohlf 2000, Zar 1996). Individual weight and survival were analyzed, and least squares means were estimated using PROC GLM in SAS statistical software version 9.2 (SAS Institute 2009).

Family was entered as a random effect, block as a fixed effect, and block by family interaction as a random effect. Appropriate F-statistics were generated using the TEST option in SAS PROC GLM for mixed effects models. Data adjustments, histograms, cumulative normal probability charts, spread versus level plots, and regressions were performed using Excel 2007. Within the subtidal site, individual weights were adjusted for differences among blocks by deviation, and then for differences among nets by calculating standard scores (Sokal and Rohlf 1997) within each net. These scores were then ranked from high to low among all nets and all blocks. Walk-back selection (Doyle and Herbinger 1998) was then employed from highest to lowest score. For the intertidal site, standard score was calculated within each block, and all individuals were sorted as they were at the subtidal site.

Results

General Linear Models

Factorial, between subjects, univariate GLM (ANOVA) were conducted to assess the effect of family on individual weight and survival, as well as

environmental (nuisance) factors including block and net. These GLM produced least squares estimated means, adjusted for nuisance factors, which were later used in regressions in order to compare the two methods (mixed versus separate). Non-significant factors were removed from the models ($p>>0.05$). Family and net were random effects, and block was a fixed effect. A summary of GLM output detailing the models used to estimate least squares family means is presented in Table 4.1.

For the mixed treatment, at the intertidal site, there were significant effects of block [$F(3, 1050) = 21.75$, $p < 0.0001$] and family [$F(47, 1050) = 6.20$, $p < 0.0001$] on individual weight (Table 4.1). For the mixed treatment at the subtidal site, there were significant effects of net [$F(11, 1063) = 4.19$, $p < 0.0001$] and family [$F(47, 1063) = 3.26$, $p < 0.0001$] on individual weight. For the separate treatment at the intertidal site, there were significant effects of block [$F(3, 141)$ 14.84, $p < 0.0001$] and family [$F(47, 141) = 2.13$, $p = 0.0004$] on individual weight . The block*family interaction was also significant [$F(141, 192) = 2.11$, $p < 0.0001$]. For the separate treatment at the subtidal site, there were significant effects of block [$F(3, 320)$ 13.60, $p < 0.0001$] and family [$F(47, 320) = 9.47$, $p < 0.0001$] on individual weight. For the mixed treatment at the intertidal site, family [$F(47, 141) = 2.15$, $p = 0.0003$] was the only significant factor affecting survival. For the mixed treatment at the subtidal site, there were significant effects of block [$F(3, 141) = 7.83$, $p < 0.0001$] and family [$F(47, 141) = 2.95$, $p < 0.0001$] on survival. For the separate treatment at the intertidal site, family [$F(47, 141) = 1.97$, $p = 0.0013$] was a significant predictor survival. The block*family interaction was also significant [$F(141, 192) = 1.51$, $p = 0.0039$]. For the separate treatment at the

subtidal site, there were significant effects of block [$F(3, 320) = 33.79$, $p < 0.0001$] and family [$F(47, 320) = 3.75$, $p < 0.0001$] on survival.

Least squares estimated family means for individual weight can be found in Appendix Tables B.1. and B.3., and for survival in Appendix Tables B.2. and B.4. Probability values for all mean estimates are <0.0001. Means are sorted by family number from low to high, and by value from high to low, respectively.

No significant relationship between survival and growth for either the mixed or separate treatment was found in either the subtidal (mixed $r = -0.115$, $p=0.440$, $n=48$; separate $r = 0.043$, $p=0.770$, $n=48$), or intertidal site (mixed $r = 0.017$, $p=0.908$, $n=48$; separate $r = -0.208$, $p=0.158$, $n=48$). Within the mixed treatment, at the subtidal site, both block [$F(3, 25) = 7.31$, $p = 0.0039$] and net [$F(11, 25) = 4.14$, $p = 0.0016$] were significant factors affecting harvest bag density.

Correlation comparison of family trait means between treatments

Pearson's correlation coefficient was calculated to assess the degree of similarity of family means for survival and growth, between the mixed- and separate-family rearing methods using the "fisher" command with PROC REG in SAS 9.2. Fisher's *z*-values were used to calculate 95% confidence limits and p-values. Results are presented in Table 4.2.

Mean family individual weight for the mixed-family treatment was a significant predictor of mean family individual weight in the separate family treatment in the subtidal site ($r = 0.754$, $p<0.0001$, $n=48$, Figure 4.1) and at the intertidal site ($r = 0.728$, $p<0.0001$, $n=48$, Figure 4.2). Averaging family mean

individual weight over the two sites strengthened the relationship between the mixed and separate family treatments for this trait (r = 0.817, p<0.0001, n=48, Figure 4.3).

Mean family survival for the mixed family treatment was also a significant predictor of mean family survival for the separate family treatment at the subtidal site (r = 0.659, p<0.0001, n=48, Figure 4.4), but not at the intertidal site (r = 0.136, p=0.360, n=48, Figure 4.5). Although the removal of five outlier families made this relationship significant (r = 0.489, p=0.001, n=43), solid justification for outlier removal could not be established, and therefore, the outliers remained in the correlation summary in Table 4.2.

Selection index

A selection index of $y=0.5x + 0.5z$ was calculated for the subtidal site, where x is standardized LS mean individual weight and z is back-transformed, standardized LS mean survival (Figure 4.6). Family means were standardized according to the equation, $z = (x - \mu)/\sigma$, where z is the standard score, x is one family mean, μ is the grand mean of the family means, and σ is the standard deviation among family means. Standard scores were used to remove the effect of the different measurement scales of each variable on the relationship between them.

The selection index values were similar between the two methods at the subtidal site (r = 0.740, p<0.0001, n=48, Figure 4.7). Selection index was not calculated for the intertidal site, due to the poor relationship between methods for survival at that site (Figure 4.5).

We compared the results of simulated truncation selection between the two different methods using mean trait values of each family. The top 8 ranked families (top 15% of families) were compared between methods at each site, by index, and by individual weight and survival. For the index, six of the same families (47, 76, 8, 15, 25, and 19) were present in the eight top-ranked families of both the separate and mixed treatments at the subtidal site (Table 4.5, Figure 4.6). Agreement was not as close for survival or growth at either site; four of the same families were present in the eight top-ranked families for individual weight, at the subtidal site, and four at the intertidal site. Five of eight were the same for survival at the subtidal site, and two of eight at the intertidal site.

Walk-back

Oysters from all 48 families were encountered in the subtidal site after walk-back sampling the heaviest 528 individuals (out of 1126, Figure 4.8), while it took 326 oysters to encounter individuals from each family at the intertidal site (out of 1080, Figure 4.8).

In order to compare the results of walk-back (individual) selection versus selecting by family means of individual weight, individuals were sorted by their individual weight (adjusted by block and net, where appropriate, by methods discussed earlier) and a sample of 528 individuals was selected from the heavy end of the distribution at the subtidal site, and 326 from the intertidal site. The frequencies of each family were determined, and families were ranked by their frequency within each sample. They were then compared to the ranked family mean individual weights using a Spearman Rank Correlation. The comparison at

the subtidal site yielded a Spearman's rho of 0.917 (df=46, p<0.0001, Figure 4.9), and at the intertidal site, it was 0.744 (df=46, p<0.0001, Figure 4.10).

MBP data analysis – components of yield

MBP cohort means from two generations of data in two separate lineages, which included six cohorts (Cohorts 11, 14, 15, 16, 18 and 20) were analyzed using OLS regression and a generalized linear model with generalized estimating equations (GEE) for clustered data, to examine the relationships among survival, individual weight, and yield across these cohorts. Parameter values were the same between the OLS and GEE methods, and significance p-values were very similar. Survival (arcsine transformed) was a significant predictor of yield (Table 4.4, Figures 4.11-4.13), with an R^2 of 0.623, (OLS p=0.007, GEE p=0.001), demonstrating that survival accounted for 62 percent of the variation in yield across these cohorts. Individual weight was not a significant predictor of yield (OLS p=0.176, GEE p=0.095), although it showed an R^2 of 0.216, similar to the Cohort 18 data. Individual weight was not correlated with survival (r = -0.005, p=0.994).

MBP LS family means from Cohort 18 (the cohort used in this study) with families reared separately were used to determine whether survival or individual weight was more important in determining yield (Table 4.5, Figures 4.14 - 4.17). In this data, yield was measured directly as bag weight (kg). Survival (arcsine transformed, as before) was a significant predictor of yield at both the subtidal and intertidal sites (subtidal R^2=0.602, p=$1X10^{-11}$; intertidal R^2=0.542, p=$5.07X10^{-10}$) using OLS regression. Thus, survival accounted for 60 percent and 54 percent of

the variation (Sokal and Rohlf 1996) in yield at the subtidal and intertidal sites respectively. Individual weight was also a significant predictor of yield at both sites (subtidal R^2=0.366, p=2X10^{-6}; intertidal R^2=0.215, p=0.0005). Individual weight accounted for 37 percent, and 21.5 percent of the variation in yield at the subtidal and intertidal sites, respectively.

Discussion

Rearing oysters of different families mixed together yielded very similar results to rearing them separately when comparing family mean individual weight, survival and yield index at two sites. This suggests it is unlikely that genotype-dependent associative effects, such as exploitative or interference competition, or resource partitioning (Bertness 1989, Brichette 2004, Frechette 1992, Griffin 1996, Jarayabhand and Newkirk 1989, Lohse 2002, Muir 2005, Wade 1978), are of high importance in the Pacific oyster in the commercial production environment. Differences in estimation of means between the two methods, together with low sample size for survival estimation, and high within-plot environmental heterogeneity at the intertidal site could easily account for the majority of discrepancy in family means between methods.

Both net and block were highly significant predictors of bag density in the mixed-family, subtidal treatment, suggesting that both survival and growth were directly affected by net and block. In the MBP planting, net was not recorded and could not be used as a factor in the analysis. Judging by these results, it would be advantageous to include net as a factor in the separate-family method in the future.

The confounding of family-specific growth and survival due to density dependence, which creates common environmental effects for individual weight, is a concern about rearing families separately for selective breeding. However, this was not the case in either method in this experiment, as stated in the results. Family mean survival was not correlated with family mean individual weight in either method.

For survival at the intertidal site, there was a highly significant interaction between block and family in the separate treatment, but this interaction was non-significant in the mixed treatment. This is not surprising, due to the inherent differences in the experimental designs of the two methods. The intertidal site was very muddy, due to silt runoff that coincided with recent clear-cut logging directly above the site, and survival estimation for both methods likely suffered as a result. The degree of siltation varied greatly and unpredictably along the beach. In the separate treatment, if a bag became buried, and the inhabitants of the bag died or grew slowly due to the silt, it greatly affected the mean for that family (eight bags per family overall). However, in the mixed treatment, it affected the mean of all families more evenly (although randomly), since there was one individual from each family in each bag, and since sample sizes in the mixed treatment were small (8 individuals per family overall), it resulted in high estimation error across all families. These fundamental differences in design in this heterogeneous environment were likely responsible for the relatively large differences in estimated means between the two methods for intertidal survival, rather than a

genotype by environment interaction between mixed and separate-family environments.

Low sample size for survival estimation also hampered the mixed treatment's ability to account for environmental variation within the subtidal site. Because of the low sample size, net could not be included as a factor in the analysis of survival at the subtidal site, as there were not enough degrees of freedom available (although this factor was important for individual weight at harvest) and thus, the estimated means were not adjusted accordingly. Instead, the effects of net were combined with the block and family effects. Since the design was well balanced, this is not catastrophic, but the correlation between mean family survival in the mixed and separate treatments would likely be significantly stronger with a higher sample size (i.e. within-block replication) in the mixed treatment. The sample sizes were n=400 individuals per family over eight bags for the separate family method, versus n=40 per family over eight bags for mixed in the subtidal (n=32 per family for the intertidal). The difference in precision is evident in the standard errors around the LS mean estimates (Appendices 2.A.-2.D.), which are approximately twice as large for mixed than separate, for both survival and growth, except intertidal individual weight, which were very close. In the subtidal mixed treatment, there were 10 individuals from each family planted per block, so the precision was in units of 10 percent, compared with the separate family design which had 50 animals in each bag, for a precision of 2 percent, and two bags per block, which provided eight replicated point estimates of survival, while the mixed treatment only had four. This means four estimates with 10 to

12.5 percent precision each in the mixed treatment, versus 8 estimates with 2 percent precision in the separate. Fixed funds for genotyping lead to a practical limitation of approximately 2400 individuals. This demonstrates that estimation of survival, an aggregate trait, requires much higher sample sizes than does an individual trait, such as growth. For estimating survival, the mixed method will range along a continuum between being much more costly than the separate method, or much less precise in its estimation of family means.

The sample sizes of the mixed treatment were still quite adequate for estimating individual growth, either as family means or using a walk-back approach. Doyle and Herbinger (1994) introduced their version of mixed family selection to aquaculture, coining the term "walk-back selection". Walk-back selection entails harvesting a mixed-family group of organisms, measuring them for an individual trait, sorting them by that trait from high to low, and then "walking back" the phenotypic distribution until replacements from enough families have been selected to produce another generation. Since then, walk-back selection has been explored with several aquaculture species including Atlantic salmon (Herbinger et. al. 1999), Norway lobster (Streiff et al. 2004), African catfish (Volckaert et al. 1999) and European lobster (Jorstad et al. 2005).

Employing walk-back selection for individual weight in this study produced very similar choices of families as selection by family means (Spearman's rho, subtidal = 0.917, intertidal rho = 0.744). Also, individuals from all families were encountered using walk-back selection for individual weight within a few hundred samples, contrary to fears stemming from previous research

at early life history stages (Taris et al. 2005) that just a few, large families could dominate the upper end of the size distribution. The initial discovery curve was quite steep, with an average of 27.5 families discovered in the first sample of 48 heaviest individuals (Figure 4.8). This result is due to two related factors: highly overlapping family distributions of individual weight, and the fact that survival and growth were uncorrelated in this experiment. Had they been strongly correlated, the slope of the discovery curve would likely have been much less steep. The results also showed that a sample of a few hundred produced a reasonable number of individuals from enough different families to minimize inbreeding ($F<0.0625$) when producing another generation.

Cost comparison among methods

Figure 4.18 shows a comparison of estimated costs for separate-family ("Separate"), mixed-family ("Mixed"), and walkback/mixed-family ("Walkback") breeding designs for the Pacific oyster, based on costs for consumables and labor, leaving out travel (in effect, assuming a local field site, such as Yaquina Bay is to HMSC) and overhead costs for simplicity of comparison.

The Mixed design for estimating survival and individual weight was most expensive ($32,407), even though it has a lower sample size, and an accompanying lower expected accuracy, as well as precision of survival estimation (5% without replication, or 10% with replication) than the Separate design (2% with replication), whose overall cost was estimated at $27,147. However, the Walk-back design, which estimates only individual traits via walk-back selection had the lowest estimated cost ($18,726).

The distribution of costs was quite different among the three designs. The Separate design spent most (53%) on hatchery/nursery and field expenses (39%), and little (8%) on genotyping, while the Mixed design spent most (49%) on genotyping, followed closely by hatchery/nursery expenses (44%), and least on field expenses (6%). The Walk-back design spent the most on hatchery and nursery (77%), and comparatively little on field (11%) and genotyping (12%) expenses.

In this comparison, overall average field survival was assumed to be 50%. This is important for the Mixed design, because since genotyping costs of this design are determined directly by the number of individuals genotyped; all the individuals in a set number of bags must be genotyped in order to estimate survival. Thus, if one uses the average field survival from this experiment, 69%, the costs of the Mixed design rise from $32,407 to $38,183, by 18% (Table 4.6.b.) This increase in survival makes the Mixed design cost 41% more than the Separate design, while at 50% survival, the Mixed design cost 19% more than Separate. The number of oysters to be genotyped for the Separate and Walkback method is unrelated to survival, and thus so is the cost.

Also, the hatchery and nursery costs to produce 50 families were assumed equal among designs for two main reasons. First, oysters from different families are to be reared separately through the hatchery and nursery, and only mixed as spat (juveniles) since we determined in Chapter 3 of this dissertation that families mixed any earlier than this would no longer be evenly mixed when they grew to planting size; this would necessitate additional genotyping of large samples in

order to determine family composition at planting, which would be cost-prohibitive. Second, for large numbers of different families, the number of families produced is the primary determinant of cost, within the range of numbers of spat required to carry out these three breeding designs. The final number of spat in each silo, whether it is eighty (for Mixed or Walk-back) or four hundred (Separate), would be insignificant in determining hatchery and nursery cost.

Both the Separate and Mixed designs assume selection is carried out for survival in addition to individual weight; survival is an aggregate trait, estimated as a proportion, and thus requires at least ten times as many individuals to estimate it with any precision. Accordingly, the Separate design has 400 bags of 50 oysters each, approximately equal to the same treatment in current study. This provides a precision of survival estimation of two percent, and two replicates per block. It provides a total of eight estimates of survival and mean family individual weight for each family. Individual weight under this design is estimated by dividing bag weight by live oyster count within the bag.

Table 4.6 summarizes the experimental aspects of the Separate, Mixed, and Walk-back designs as they are used in this study; this table shows the blocking and replication structure for each design, by trait. The Mixed design assumes a planting of 80 bags of 50 oysters each (one oyster from each family within each bag), with two replicates of ten bags each per block (20 bags per block). This results in a precision of survival estimation of ten percent (the same as the present study for the mixed treatment) and two replicates per block. The mixed treatment in the current study had no replication within block for estimating survival, which

prevented estimation of a block by family interaction (which was present in the intertidal separate treatment), and likely contributed to a poor relationship between survival in the mixed and separate treatments in the intertidal (intertidal survival $r = 0.137$). Therefore, replication was added to the Mixed design for the cost comparison. Alternatively, one could increase precision to five percent, consider all 20 bags in each block as one replicate for estimating survival, and lose the ability to estimate a block by family interaction, for the same cost. Four thousand oysters are planted under the mixed design, and (as stated earlier) an overall survival of fifty percent is assumed for all three designs. For the Mixed design, this means that two thousand oysters would be genotyped for the level of precision of survival estimation discussed earlier. This makes this a conservative cost estimate, given that the level of average mortality has often been lower than this, which would mean more oysters to genotype in the Mixed design.

The Walk-back design assumes selection is performed for only individual weight, which enables low genotyping costs for only the 200 largest individuals (plus 100 parents), and enables a low total population of 50 bags times 50 individuals per bag. The Separate design also genotypes only the individuals selected for breeding (300 in this design).

Estimated cost structure for the three designs is presented in Table 4.7. Genotyping costs were calculated as $7.60 per sample, including extraction, biopsy and tagging costs. We endeavored to make genotyping as economical as possible for this cost comparison. Genotyping costs for electrophoresis were calculated using the OSU rate at HMSC of $0.52 per sample (excluding ladder and

formamide), coloading four microsatellite markers. This was quite inexpensive as compared with the Oregon State University Core Facilities genotyping service for non-OSU personnel, of $5.30 per sample. Consumables cost of extraction was estimated as approximately $0.50 per sample, using the low-cost method of Ivanova et. al. (2008); this is quite low compared with the approximately $2.00 per sample cost of Quiagen extraction kits, which are widely used. All labor costs were estimated as $24 per hour (including non-wage costs). Field costs were calculated from typical MBP costs by total hours spent and equipment costs, then divided by number of bags, and applied to the mixed and walk-back designs on a cost per bag basis. Hatchery and nursery costs were estimated from MBP, and were assumed to cost the same for each design, due to similar labor and equipment needed within the range of number of spat needed for the three designs.

Breeders should consider their goals carefully when deciding which method to use. Costs within each method are apportioned differently, and each method has strengths and weaknesses. The separate-family method is efficient at making additive genetic progress, preventing inbreeding, accounting for macro-environmental variation, and selecting on both aggregate and individual traits. Although some degree of common environmental effect is possible through density dependent growth, if stocking density is set beneath the threshold of density dependence, this can be avoided. The relevant density dependence dynamics for growth could be revealed through one or two experiments. The separate-family method is not limited to among-family selection. Additional, within-family selection can easily be applied by using the heaviest individuals

within a particular growing unit and correcting individual values for environmental factors. Combination selection has been determined as the most efficient for making long-term additive genetic progress by many authors. An additional consideration is that mixed-family rearing would not be economically viable for a large-scale breeding program (such as MBP), which needs to maintain a broodstock repository of specific families for amplification by industry, since this would require biopsying, genotyping and parentage assignment for very large numbers of animals.

In a survey of west coast oyster growers (Langdon et al. 2001), yield as whole bag weight was identified as the most important trait for improvement by selective breeding. Thus, an important issue to consider when choosing a method to use for breeding Pacific oysters on the West coast of the U.S. is that survival has been demonstrated as more important in determining yield than individual weight. Data from the MBP, collected from ten field trials, using six cohorts (including the cohort analyzed for this study, C18), at six different sites on the west coast, with full-sib families from two different lineages, clearly demonstrates that survival was responsible for 62 percent of the variation in yield (bag weight in kg, Table 4.4, Figure 4.15), and that although individual weight was a significant predictor of yield using GEE ($p=0.0434$), it was not significant in the OLS ($p=0.171$), where the R-square was 0.2158, implying that at it was responsible for 22 percent of the variation in yield (Table 4.5, Figure 4.14).

Given that survival is the primary determinant of yield, it seems prudent to include this trait in any selective breeding program for Pacific oysters. Any trait

which one intends to make genetic improvement upon should be carefully quantified, with as much measurement precision, and accuracy of prediction of breeding value as is economically feasible (Bourdon 2000). Although we were quite conservative with estimation of genotyping cost (and this is responsible for the majority of cost in the mixed method), implementation of a mixed-family selection program that could estimate survival with a precision of either ten percent with replication, or five percent without replication, still cost significantly more (22%) than separate-family selection with two percent precision of survival estimation. If survival is still not important to the breeder after considering this, then the walk-back approach was thirty percent cheaper than the separate-family method, but had no capacity to measure aggregate traits, such as survival.

Under the walk-back design, selecting the heaviest 200 or 300 oysters in our study would represent a random sample in terms of survival, since there was no significant correlation between individual weight and survival in either treatment or site in this study (Table 4.5), or in the larger analysis of six MBP cohorts across ten different field trials (Table 4.4, Figure 4.13). Selection of any significant intensity for survival would not be possible for a random sample as small as a few hundred oysters from fifty different families, as it would be impossible to estimate survival for each family with any certainty. Even in the framework of mass selection, it would only be possible to select a group of individuals from the population that belonged to families with survival of an average value that approximates the population mean. By definition, the selection intensity under this scenario is zero. One could argue that if an extreme mortality

event affected such a population, breeding the survivors would likely move the mean survival forward in the next generation. In practice, however, extreme mortality events are unpredictable and not common. Thus, it is more prudent to carry out breeding using a design where variation in survival can be estimated, and selection pressure can be applied that is in significant excess of the population mean, in order to make genetic change from virtually any field trial, even when a rare, extreme mortality event does not occur.

It is also important to consider that the mixed-family approach, as we implemented it here, using molecular markers for parentage assignment, requires either a pay-for-services arrangement, contract, or in-kind reimbursement or collaboration with someone with access to a molecular laboratory. The costs that we used in our comparison of the three methods were calculated with low service fees and consumable costs available to the University. The non-university rate for capillary electrophoresis, a significant portion of the genotyping cost, was approximately ten times as much as the university rate ($5.30 per sample versus $0.52 per sample respectively). Conducting mixed-family breeding outside of a university collaboration could be significantly more expensive than our estimates.

Finally, the mixed method appears better at dealing with very heterogenous environments, such as the intertidal site in our experiment, so it may be a good choice for those breeders limited to sub-optimal sites. Also, it allows for selection for individual traits at relatively low sample sizes and cost. However, a large, random sample must be taken to estimate survival, which would incur a sizable additional cost for genotyping those animals. If survival is important, the separate-

family method would be a more economical choice, since the minimum number of individuals needed to estimate depends on the same statistical considerations regardless of the rearing method.

Conclusions

Overall, rearing oysters of different families mixed together yielded very similar results to rearing them separately when comparing family mean individual weight and survival. This demonstrates it is unlikely that associative effects are of great importance in the Pacific oyster at this range of stocking densities. Differences in how the two methods deal with environmental variation, and estimate family means due to their experimental design, together with sample size differences (especially important for survival estimation), and high within-plot environmental heterogeneity at the intertidal site, could easily account for discrepancy in family means between methods. The mixed method was well-suited for selection by individual weight by family or walk-back selection, but would incur high genotyping costs (which vary according to average plot survival) in order to estimate survival with acceptable levels of precision and accuracy. Costs of breeding with families reared separately, or using walk-back selection with them mixed, were largely independent of oyster survival.

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Table 4.1. Summary of general linear model (GLM) output for individual weight and survival using mixed and separate rearing methods.

Method	Site	Trait	Factor	F	df-n	df-d	p
mixed	subtidal	ind. weight	model	3.390	61	1063	<0.0001
			block	1.300	3	1063	0.2737
			net	4.190	11	1063	<0.0001
			family	3.260	47	1063	<0.0001
mixed	intertidal	ind. weight	model	6.200	50	1050	<0.0001
			block	21.750	3	1050	<0.0001
			family	5.100	47	1050	<0.0001
mixed	subtidal	survival	model	3.240	50	141	<0.0001
			block	7.830	3	141	<0.0001
			family	2.950	47	141	<0.0001
mixed	intertidal	survival	model	2.080	50	141	0.0004
			block	1.000	3	141	0.3960
			family	2.150	47	141	0.0003
separate	subtidal	ind. weight	model	9.780	50	320	<0.0001
			block	13.600	3	47	<0.0001
			family	9.470	47	320	<0.0001
separate	intertidal	ind. weight	model	3.160	191	192	<0.0001
			block	14.840	3	141	<0.0001
			family	2.130	47	141	0.0004
			bxf int.	2.110	141	192	<0.0001
separate	subtidal	survival	model	5.630	50	320	<0.0001
			block	33.790	3	320	<0.0001
			family	3.750	47	320	<0.0001
separate	intertidal	survival	model	1.850	191	383	<0.0001
			block	0.230	3	141	0.8747
			family	1.970	47	141	0.0013
			bxf int.	1.510	141	192	0.0039

Table 4.2. Pearson's correlation (*r*) output for comparisons between mixed and separately reared treatments for mean family survival, individual weight, and index at two sites. Subtidal = ST, intertidal = IT, ind. wt. = individual weight.

Variable	N	Pearson's *r*	Fisher's z	95% CL		p
2-site sep ind. wt.	48	0.81712	1.14809	0.694147	0.893751	<0.0001
subindwtg	48	0.75396	0.98207	0.597918	0.854945	<0.0001
intindwtg	48	0.72767	0.92377	0.559148	0.838453	<0.0001
subtransurv	48	0.65921	0.79142	0.461522	0.794527	<0.0001
inttransurv	48	0.13565	0.13649	-0.154434	0.404208	0.3599
subindex	48	0.74043	0.95142	0.577861	0.846478	<0.0001

Table 4.3. Selection index values in the mixed and separate treatments, at the subtidal site, sorted by value from high to low.

Mixed		Separate	
family	index	family	index
47	1.282285	25	1.244326
76	1.073296	8	1.17268
39	0.998347	15	1.089819
8	0.947198	76	1.086489
15	0.750501	56	0.906812
25	0.727007	47	0.807542
20	0.660394	19	0.707748
19	0.651612	12	0.643059
42	0.606384	21	0.642265
1	0.514609	32	0.606584
16	0.426625	80	0.543045
40	0.423752	22	0.540604
46	0.416212	18	0.463783
45	0.399664	35	0.42935
22	0.375089	39	0.417233
6	0.369066	5	0.404835
35	0.368186	20	0.37724
56	0.281127	42	0.251249
18	0.274826	46	0.18582
37	0.25629	11	0.172327
12	0.255479	4	0.161455
5	0.244857	1	0.145943
21	0.197093	3	0.142106
36	0.191194	40	0.124366
49	0.167948	6	0.107179
11	0.135978	16	0.104566
24	0.071118	49	0.051128
80	-0.02266	34	-0.06081
32	-0.09553	13	-0.08476
34	-0.21269	37	-0.09411
28	-0.28589	45	-0.13045
23	-0.30913	10	-0.16754
3	-0.31794	28	-0.25494
79	-0.42077	9	-0.27986
33	-0.44183	41	-0.31699
2	-0.46035	23	-0.36204
4	-0.46283	44	-0.51384
26	-0.48384	26	-0.58349
43	-0.51105	17	-0.64277
29	-0.5434	43	-0.64794
13	-0.64121	29	-0.73789
9	-0.76479	14	-0.74606
44	-0.80316	36	-0.80599
41	-0.82066	24	-0.92686
14	-0.92751	33	-1.17825
10	-1.2595	2	-1.38426
17	-1.38205	79	-1.63299
87	-1.89933	87	-1.97771

Table 4.4. OLS (a.) and GEE (b.) regression output for survival and individual weight as predictors of yield, and the correlation between individual weight and survival (c.) in ten MBP cohorts, at 6 sites on the West coast of the U.S., for two different lineages of Pacific oysters.

a.

Response	Predictor	R^2	b	p(b)	y int.	SE(b)	95% CI	
yield	survival	0.623	4.855	0.007	-1.767	1.337	1.773	7.938
yield	ind. wt.	0.216	0.016	0.176	0.631	0.011	-0.009	0.042

b.

Response	Predictor	QIC, QICu		b	p(b)	SE	95% CI	
yield	survival	10.791	12	4.855	0.001	1.523	1.870	3.190
yield	ind. wt.	10.740	12	0.016	0.095	0.010	-0.003	0.036

c.

Variable	Variable	N	Pearson's *r*	Fisher's z	95% CL		p
mbptsurv	mbpbodwt	10	-0.005	-0.005	-0.632	0.627	0.9904

Table 4.5. OLS regression output for survival and individual weight as predictors of yield, at subtidal and intertidal sites in Yaquina Bay, Oregon, for 52 full-sib families of MBP Cohort 18 oysters (families reared separately).

Site	Predictor	R^2	b	p(b)	y int.	SE(b)	95% CI	
subtidal	survival	0.602	5.321	$1E^{-11}$	-1.202	0.612	4.093	6.550
	ind. wt.	0.366	0.027	$2E^{-6}$	0.270	0.005	0.017	0.037
intertidal	survival	0.542	2.589	$5.07E^{-10}$	-0.024	0.337	1.912	3.265
	ind. wt.	0.215	0.028	0.0005	0.737	0.001	0.013	0.043

Table 4.6. Experimental design summary of the Separate, Mixed, and Walk-back designs, as they are used in this study; this table shows the blocking and replication structure for each design, by trait.

SURVIVAL	Bags	Bags/block	Reps/fam/block	Precision	Unit
Separate	400	100	2	2%	1 bag
Mixed	80	20	2	10%	10 bags
Walk-back	80	20	-	-	-

IND. WEIGHT	Bags	Bags/block	Reps/fam/block	Measured as:
Separate	400	100	2	Ave. per bag
Mixed	80	20	20*50%=10	Individually
Walk-back	80	20	20*50%=10	Individually

Table 4.7.a. and b. Estimated costs of Separate, Mixed, and Walk-back breeding designs, by cost category. G=genotyping, F=field, and H=hatchery and nursery costs, **assuming 50% survival**.

a. Cost by category and design

Category	Separate	Mixed	Walk-back
G	2280	**15961**	2280
F	10527	2105	2105
H	14340	14340	14340
SUM	27147	**32407**	18726

b. Units and cost per unit, by category and design

Category	Separate	Mixed	Walk-back	Cost/unit	Unit
G	300	2860	300	7.60	oyster
F	400	80	80	26.32	bag
H	50	50	50	240.96	family

Table 4.7.c. Estimated costs of Separate, Mixed, and Walk-back breeding designs, by cost category. G=genotyping, F=field, and H=hatchery and nursery costs, **assuming 69% survival**, **the average between sites in this study.**

c. Cost by category and design

Category	Separate	Mixed	Walk-back
G	2280	**21738**	2280
F	10527	2105	2105
H	14340	14340	14340
SUM	27147	**38183**	18726

Table 4.8. Pearson's correlation (*r*) output summary for the correlation between individual weight and survival in the mixed, and separate-family treatments at two sites in Yaquina Bay.

Site	Treatment	N	Pearson's *r*	Fisher's *z*	95% CL		p
subtidal	mixed	48	-0.115	-0.115	-0.386	0.175	0.440
	separate	48	0.043	0.044	-0.244	0.324	0.770
intertidal	mixed	48	0.017	0.017	-0.268	0.300	0.908
	separate	48	-0.208	-0.211	-0.464	0.081	0.158

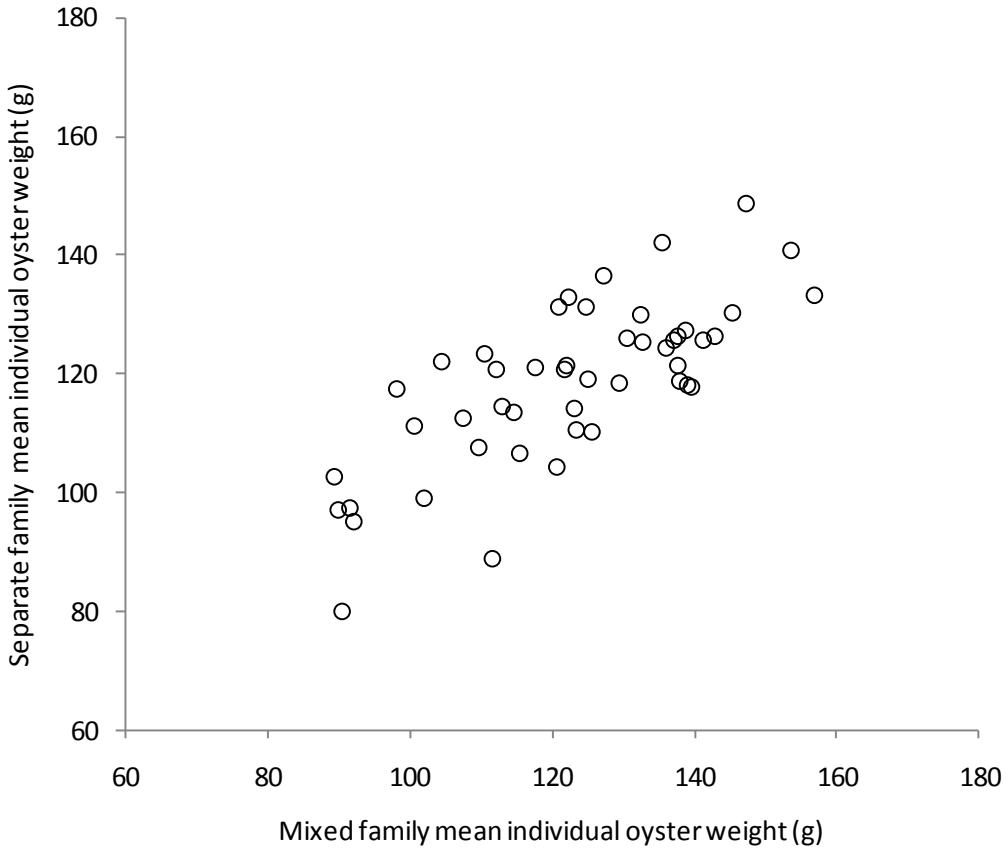

Figure 4.1. Subtidal family mean individual weights for mixed versus separate treatments (r=0.754).

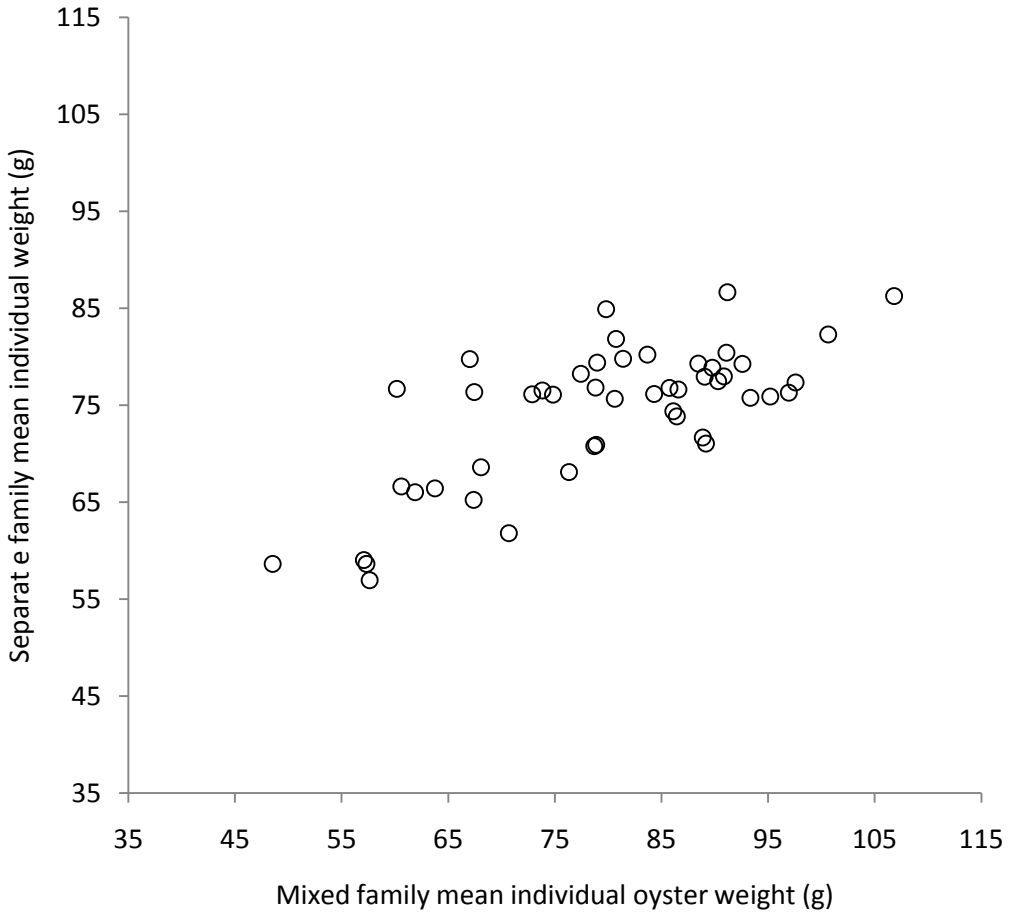

Figure 4.2. Intertidal family mean individual weights for mixed versus separate treatments.

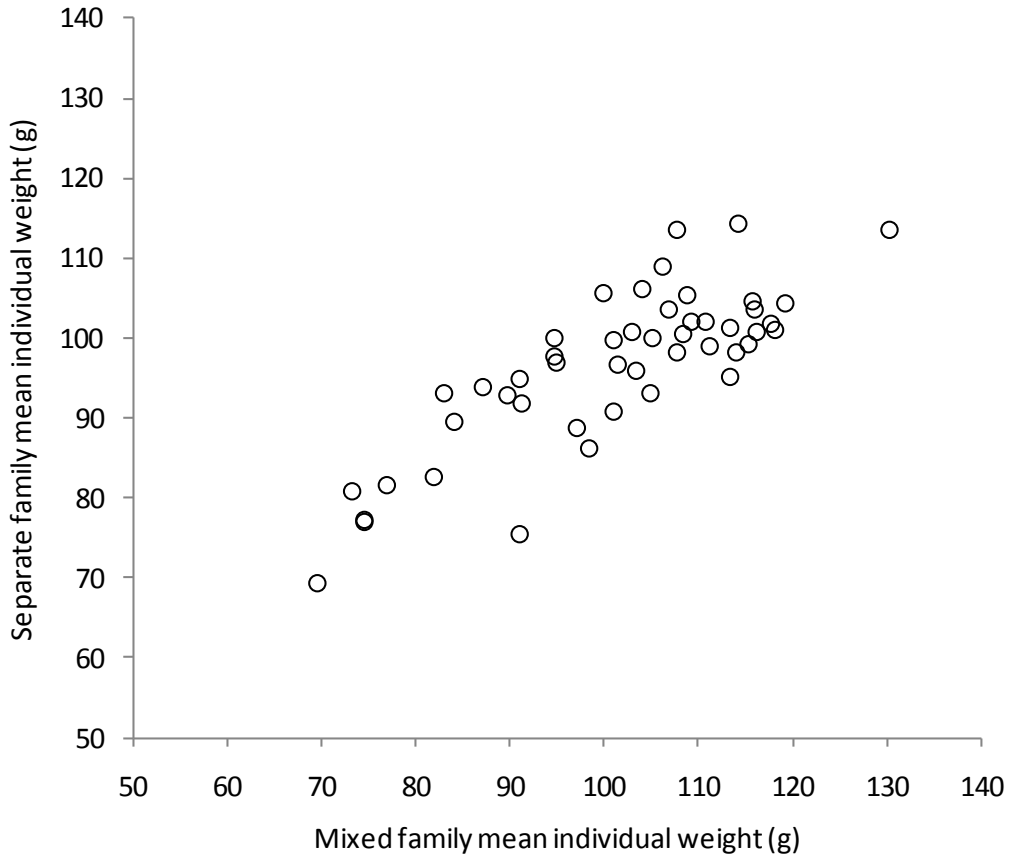

Figure 4.3. Site-averaged family mean individual weights for mixed versus separate treatments (r=0.817).

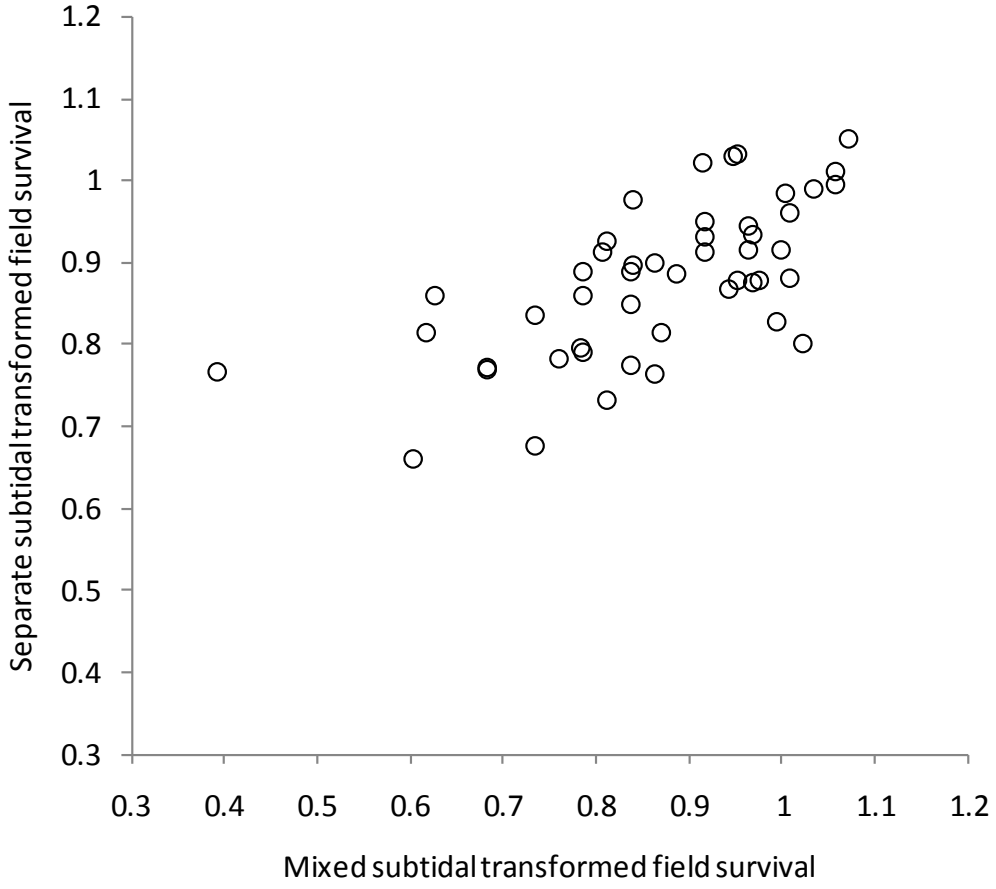

Figure 4.4. Subtidal family mean transformed survival for mixed versus separate treatments (r=0.659).

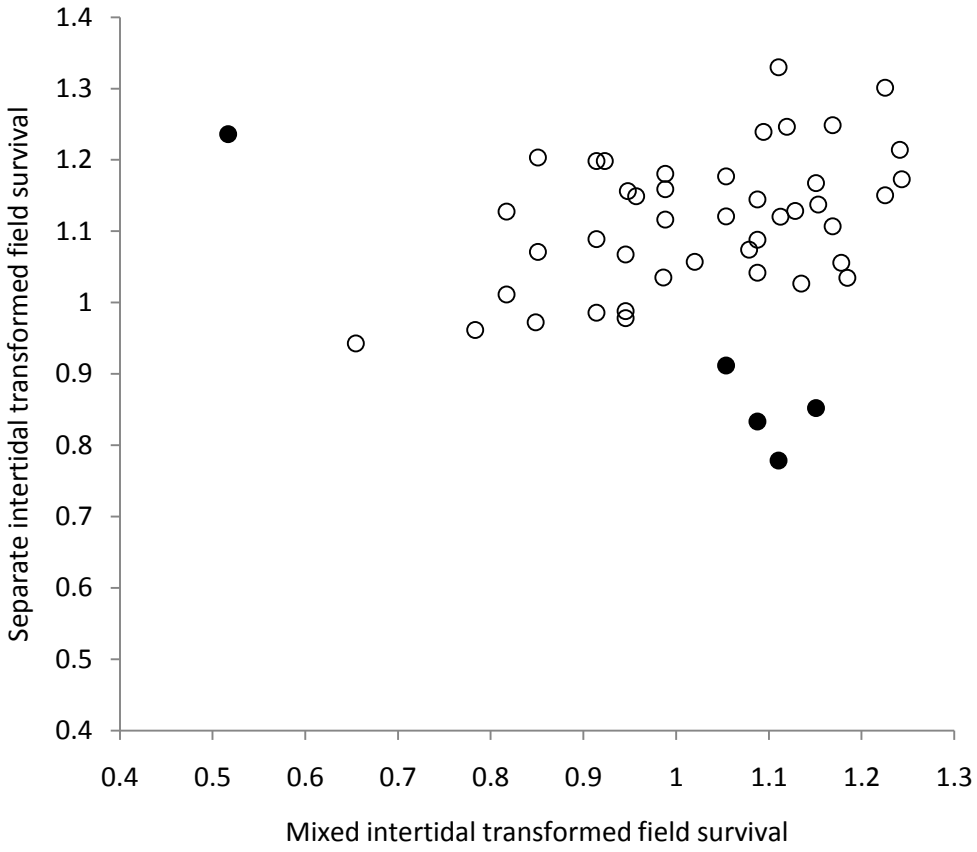

Figure 4.5. Intertidal family mean transformed survival for mixed versus separate treatments. Outliers are filled in black (r=0.136).

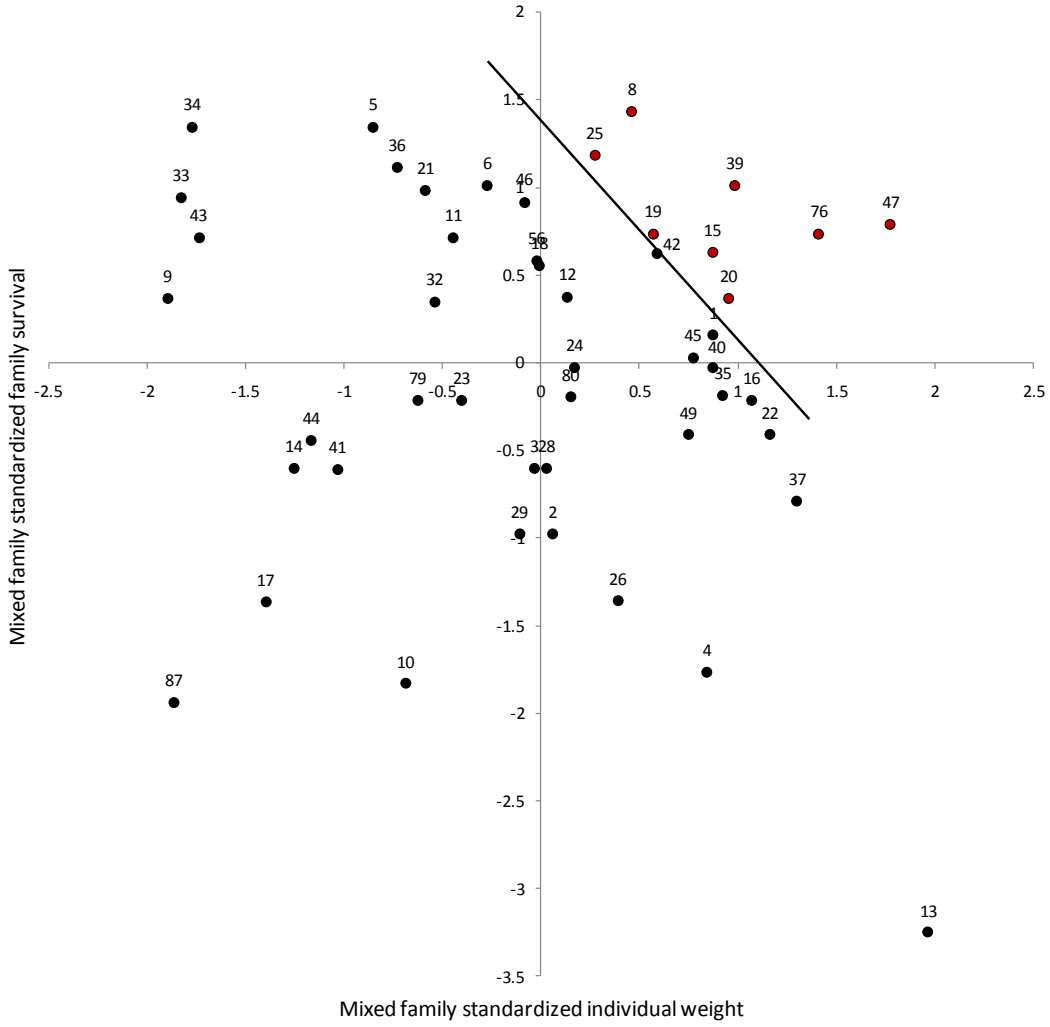

Figure 4.6. Graphical representation of among-family truncation selection by index (y=0.5 standardized individual weight + 0.5 standardized survival). Top eight ranked families (top 15%, red markers) are above and to the right of the diagonal line in the upper right quadrant. Markers are labeled with family number.

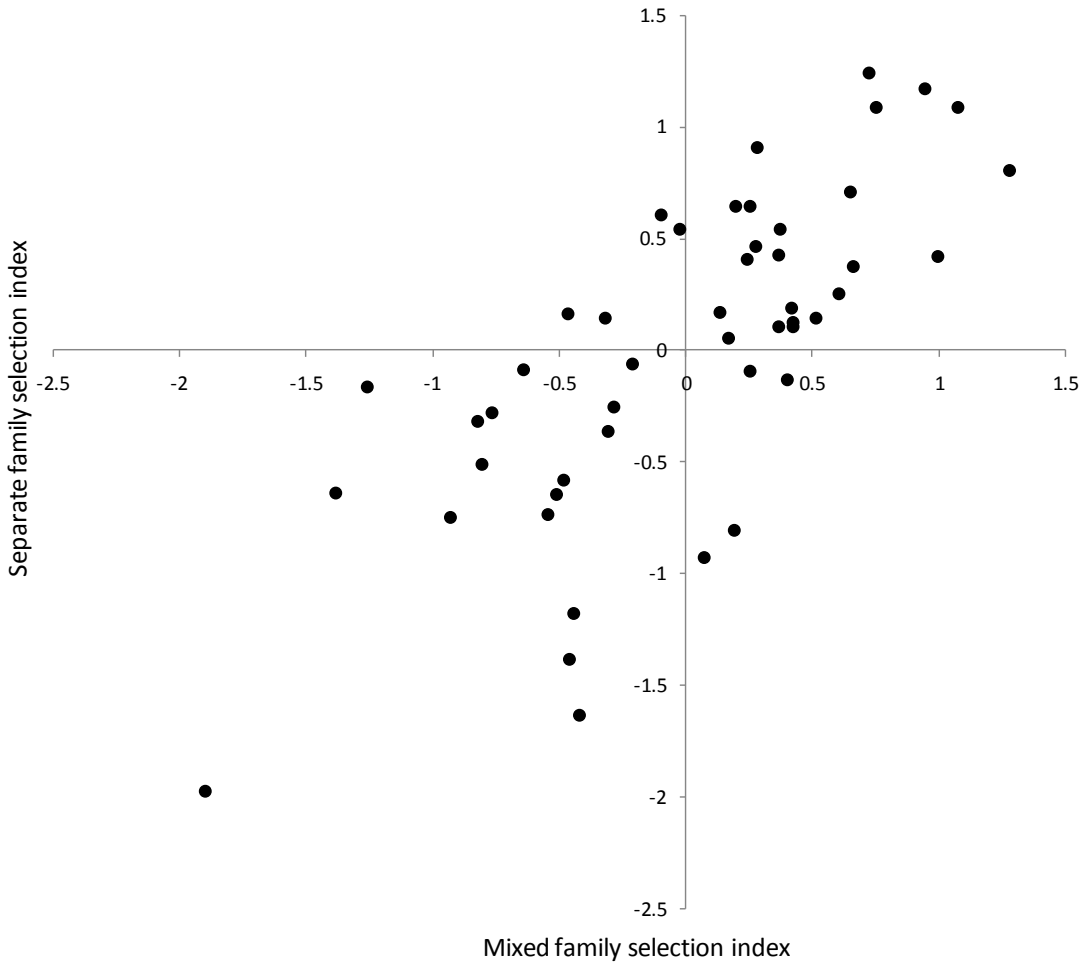

Figure 4.7. Standardized selection index for the mixed family versus separate family treatments at the subtidal site (r=0.740).

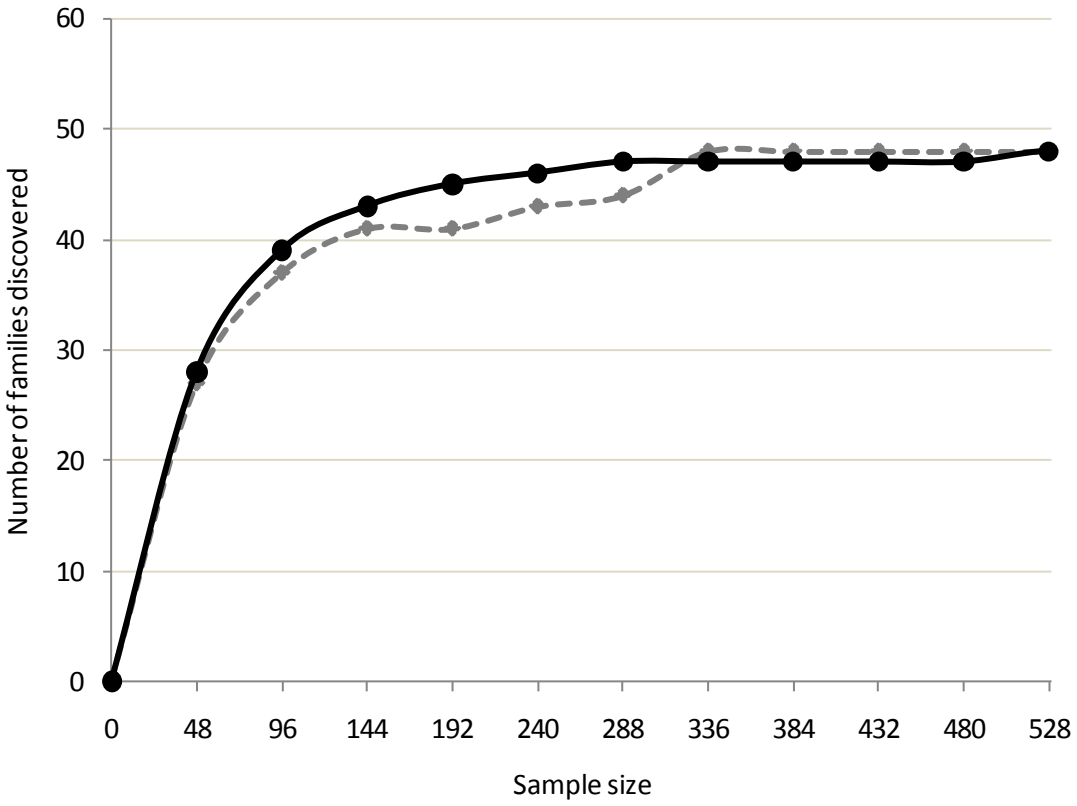

Figure 4.8. Sample size versus number of families encountered when walk-back sampling the individual weight distribution of the mixed family treatment at the subtidal (black, solid) and intertidal (grey, dashed) sites.

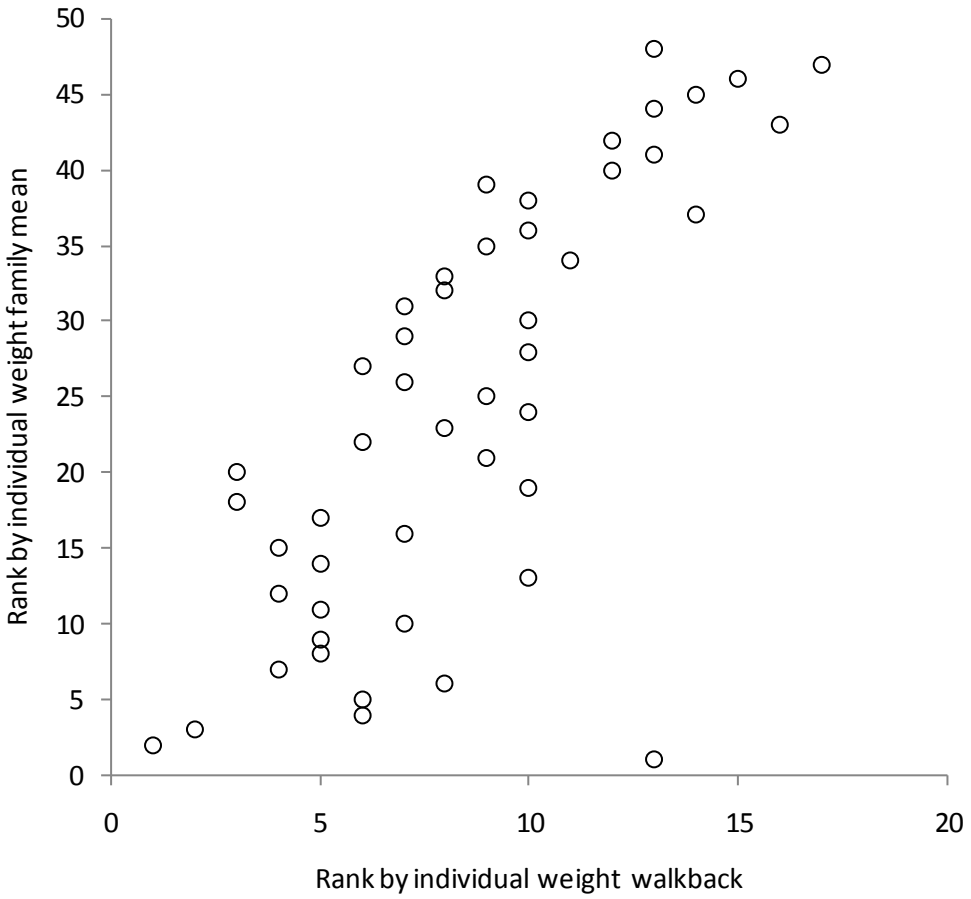

Figure 4.9. Comparison of rank by individual weight in walk-back sampling (heaviest 528) versus rank by mean family individual weight (entire data set) at the subtidal site. Spearman's rho = 0.917.

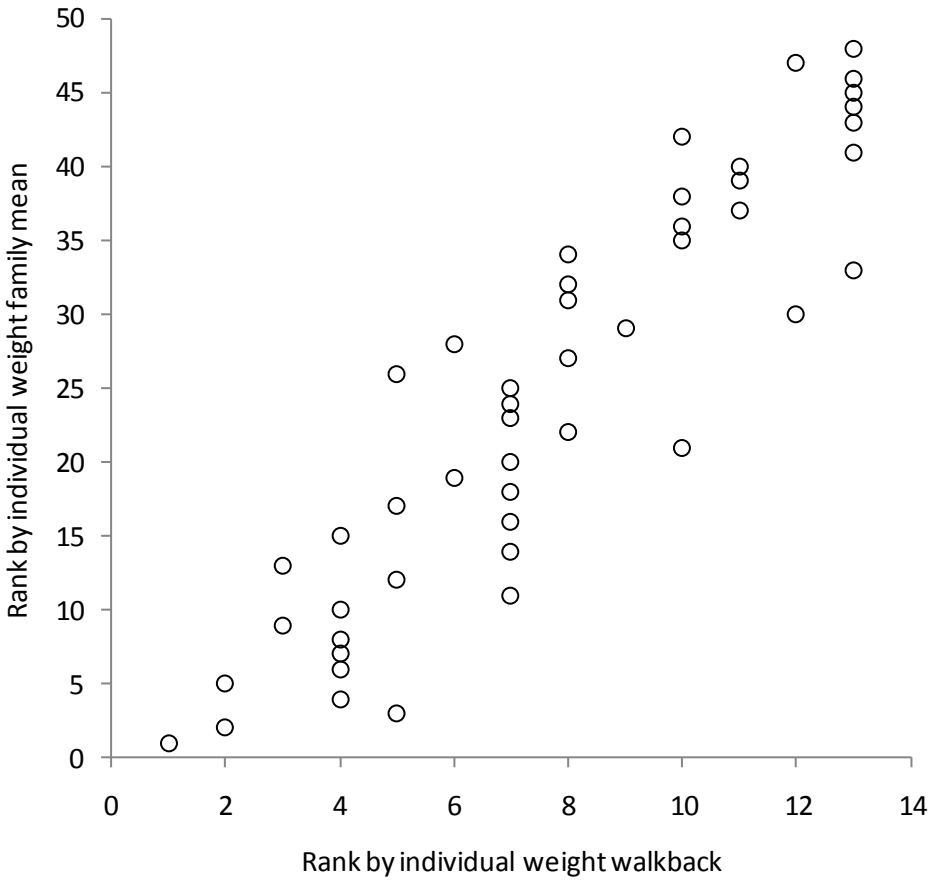

Figure 4.10. Comparison of rank by individual weight in walk-back sampling (top 336) versus rank by mean family individual weight (entire data set) at the intertidal site. Spearman's rho = 0.744.

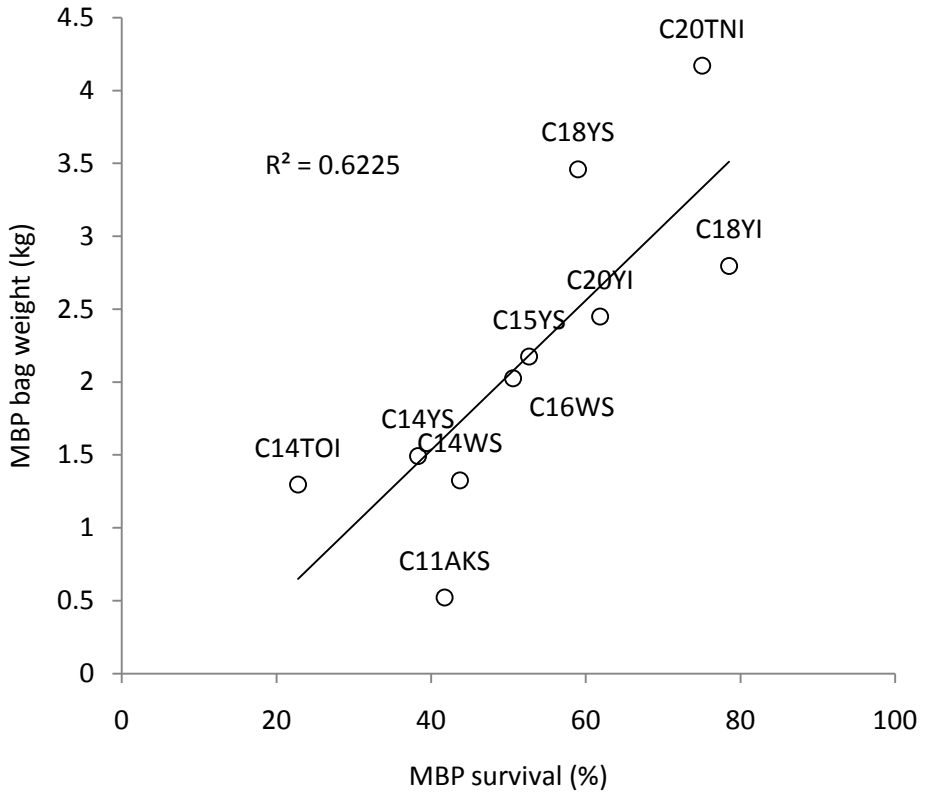

Figure 4.11. Survival versus yield (bag weight in kg) in ten MBP field trials, with six MBP cohorts, at six sites on the West coast of the U.S., for two different lineages of Pacific oysters.

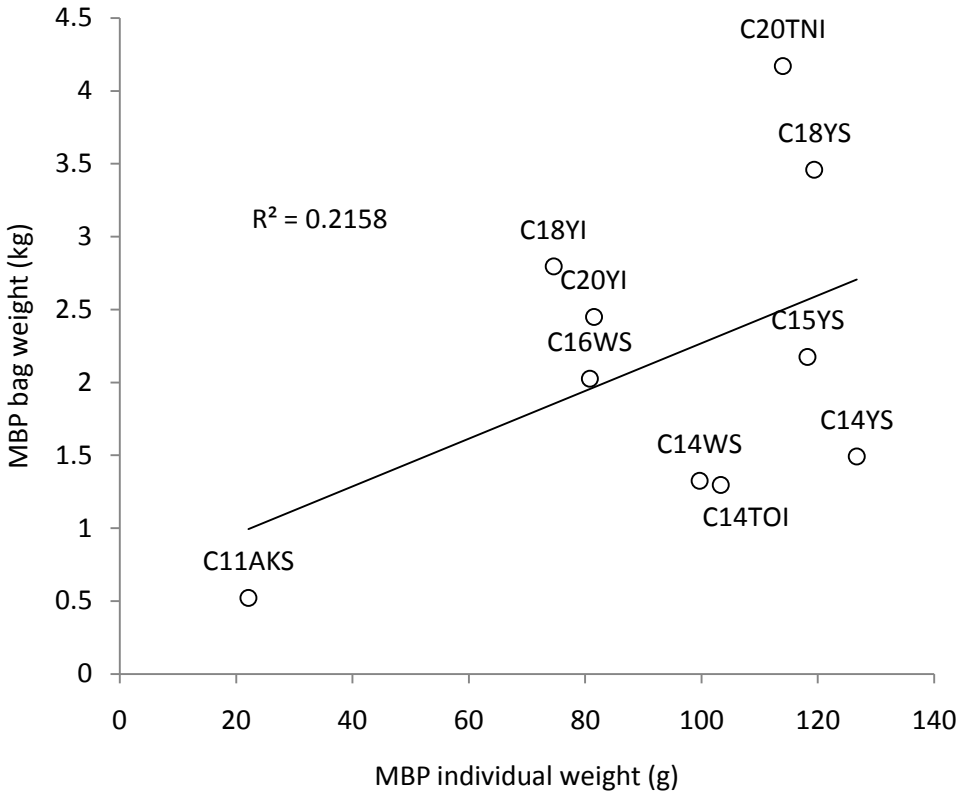

Figure 4.12. Individual weight versus yield (bag weight in kg) in ten MBP field trials, over six cohorts, at six sites on the West coast of the U.S., for two different lineages of Pacific oysters.

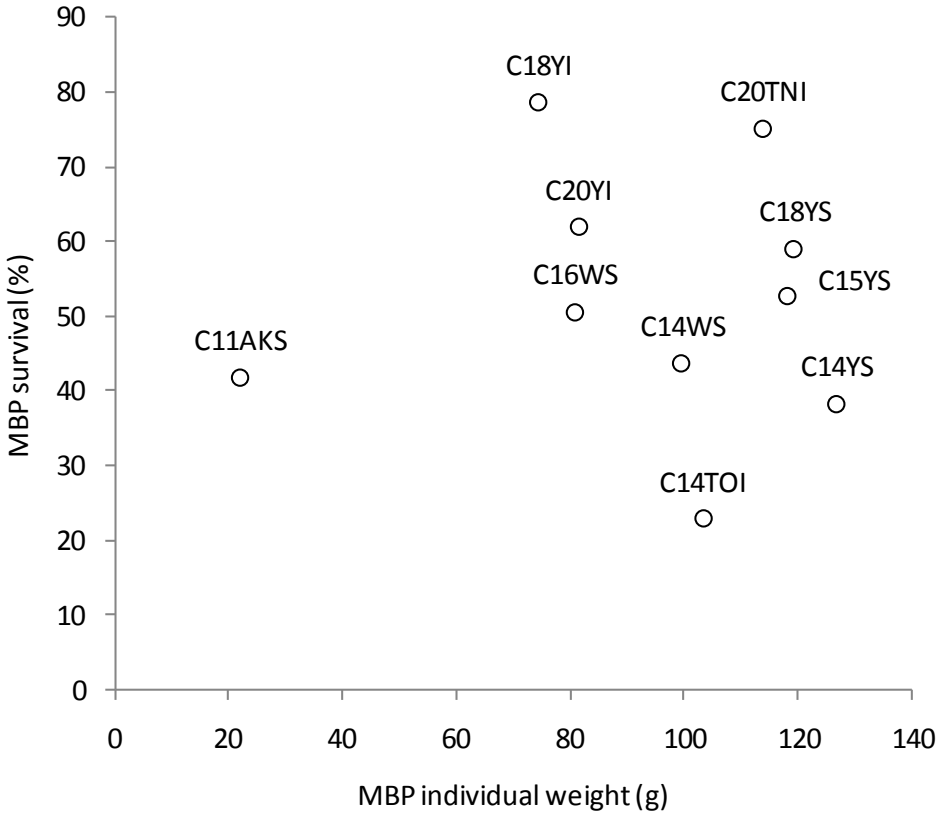

Figure 4.13. Individual weight versus survival in six MBP test sites, with six cohorts, at 6 sites on the West coast of the U.S., for two different lineages of Pacific oysters (r= -0.005).

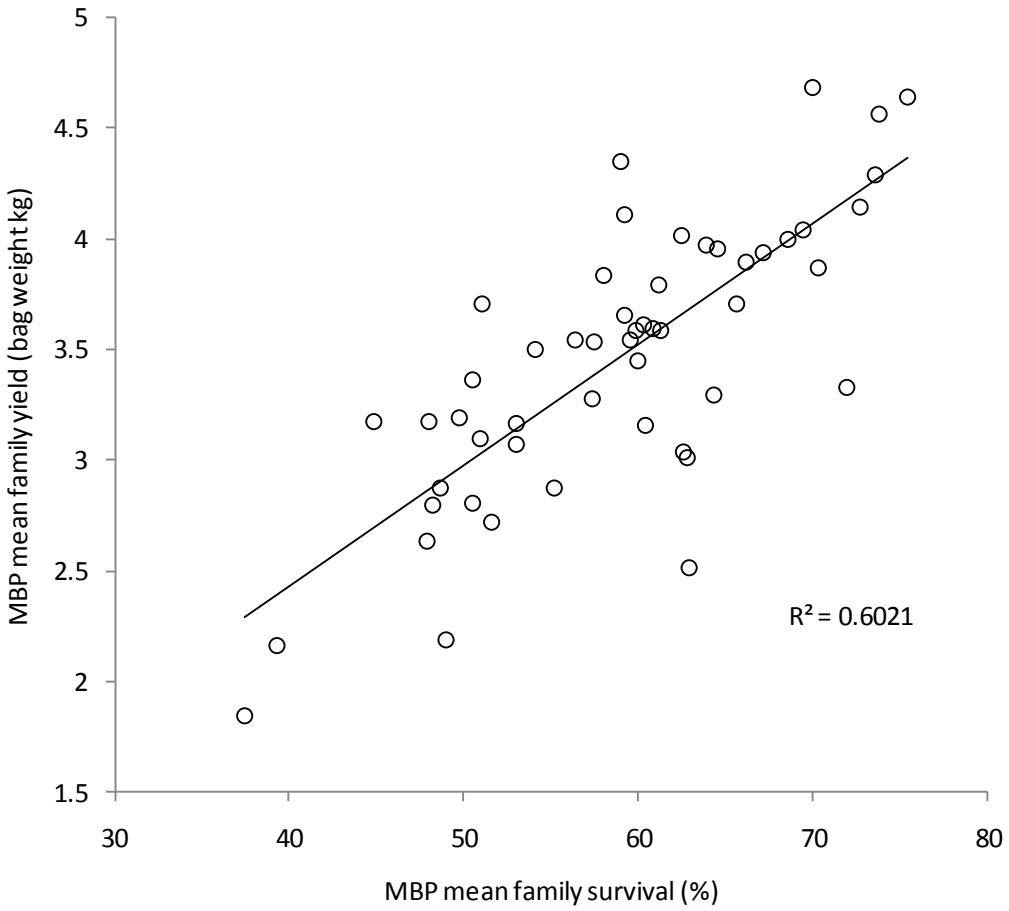

Figure 4.14. Survival versus yield of MBP Cohort 18 families at the subtidal site in Yaquina Bay (families reared separately).

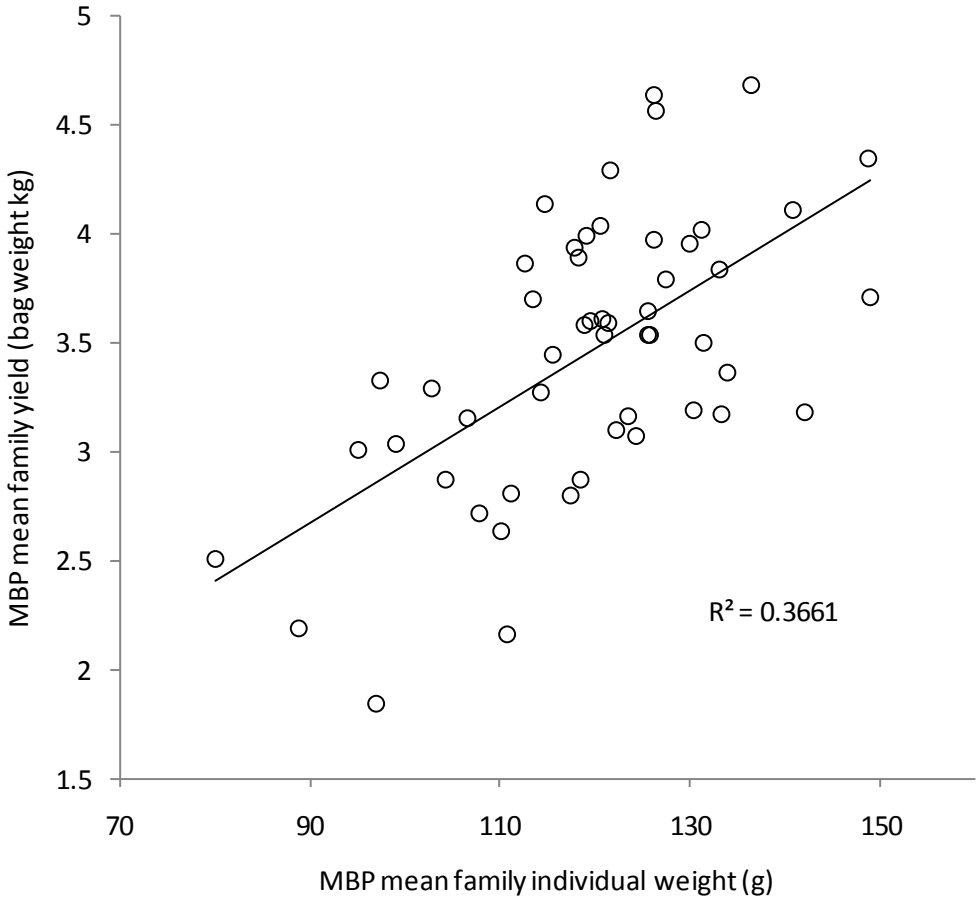

Figure 4.15. Individual weight versus yield of MBP Cohort 18 families at the subtidal site in Yaquina Bay (families reared separately).

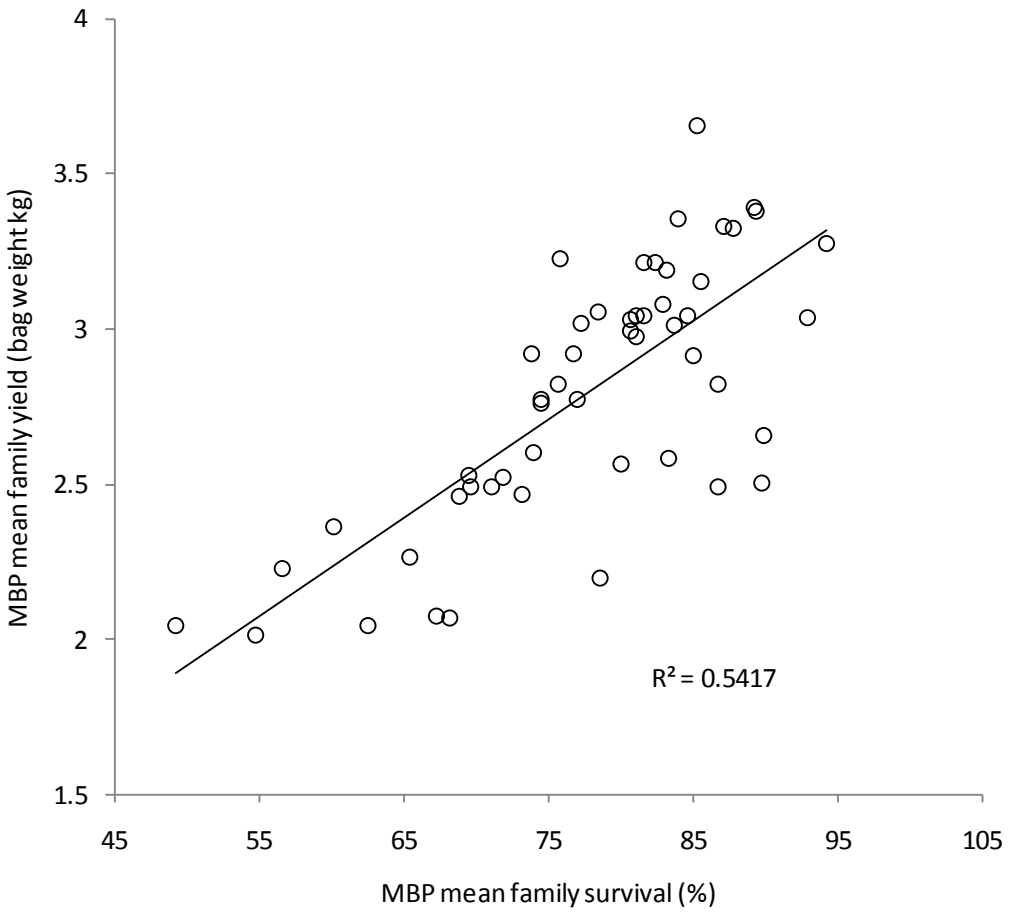

Figure 4.16. Survival versus yield of MBP Cohort 18 families at the intertidal site in Yaquina Bay (families reared separately).

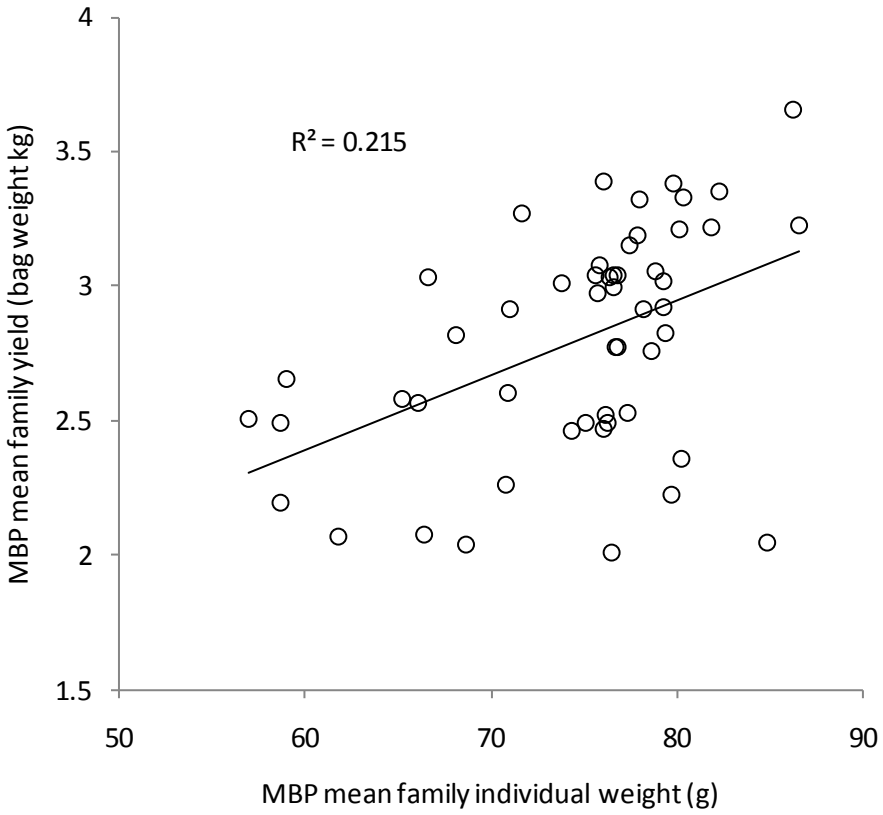

Figure 4.17. Individual weight versus yield of 52 MBP Cohort 18 families at the intertidal site in Yaquina Bay (families reared separately).

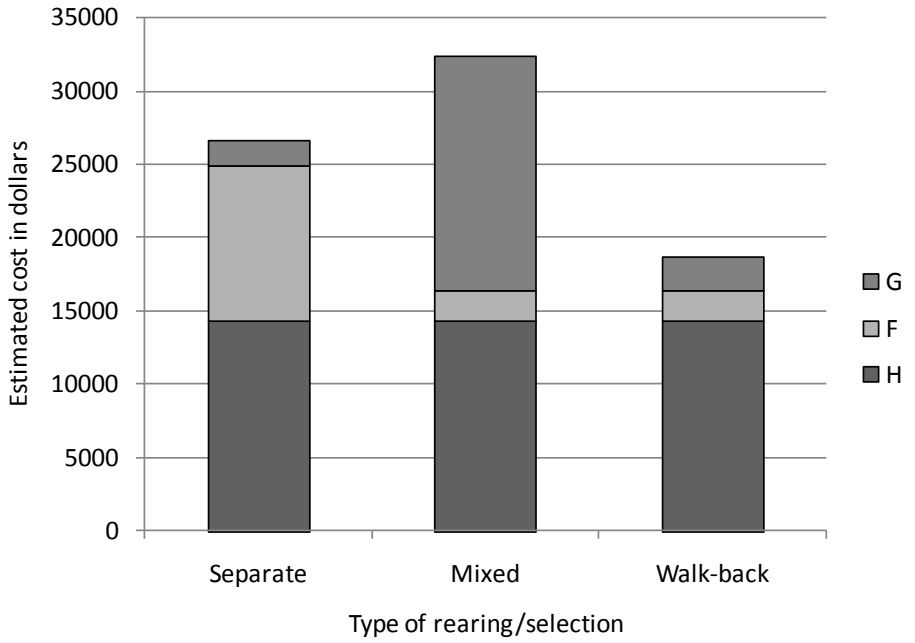

Figure 4.18.a. Cost estimates of separate, mixed, and mixed/walkback Pacific oyster breeding designs. G = genotyping costs, F = field rearing costs, and H = hatchery and nursery costs, **assuming a 50% level of field survival.**

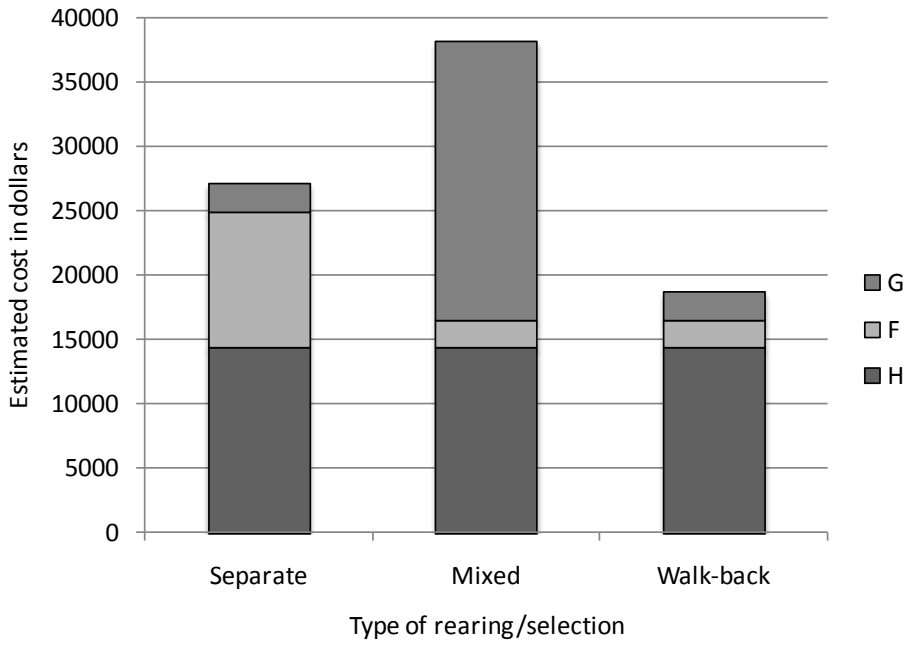

Figure 4.18.b. Cost estimates of separate, mixed, and mixed/walkback Pacific oyster breeding designs. G = genotyping costs, F = field rearing costs, and H = hatchery and nursery costs, **assuming a 69% level of field survival, the average survival between sites in this study.**

CHAPTER 5. ESTIMATION OF PACIFIC OYSTER SHELL SHAPE HERITABILITY

Abstract

Shell appearance is important to oyster producers and consumers, and as Pacific oyster shell morphology is plastic, it is often manipulated by farmers using physical methods. Changing shell shape to suit half-shell consumers via selective breeding could have economic advantages over laborious physical techniques. The heritability of any trait depends upon the genetic background and environment in which organisms are reared, and estimating trait heritability before selective breeding is a necessary part of planning of an efficient selection program. We estimated the heritability of shell shape traits using midparent-offspring regression and full-sib families in a breeding program in Yaquina Bay, Oregon. Our estimates of shell depth and shell width heritability were 0.404 ± 0.14 and 0.287 ± 0.11 respectively. These estimates are nearly equal to those from other populations of Pacific oysters in Australia, which confirms that sufficient additive genetic variation exists for depth and width to enable progress with selective breeding in this population. Heritability estimates for shell length and depth indices were not significant, suggesting that length is more plastic than depth and width, and that growing density is an important factor for shell length and related traits in the production environment

Introduction

Shell appearance is important to oyster producers and consumers (Brake et al. 2003, Batista et al. 2008, Evans et al. 2009, Sheridan 1997, Wada 1986). Brake et al. (2003) found that oyster producers' shell shape preference was primarily for oysters with higher than average ratios of depth to length, and width to length. Shell morphology of *Crassostrea* oysters is highly influenced by environmental conditions and husbandry practices (Galtsoff 1964, Jarayabhand and Thavornyutikarn 1995, Quayle 1988). Knowing this, many farmers manipulate shell shape by growing oysters in containers that encourage movement, by shaking them in their bags, or even by periodically tumbing them using rotating drums in order to break off new shell growth, called "pruning" (Quayle 1988). The fragile new shell growth is broken off primarily on the leading edge of shell length, which over time increases shell depth and width relative to length. This enhances their phenotypic similarity to the high-valued and closely related Kumomoto oyster, *Crassostrea sikamea*. Although these practices increase market value of the product, they also increase farming costs due to additional labor and special equipment. Manipulating shell shape through selective breeding instead could potentially reduce costs compared with current physical methods. In order for selective breeding to be effective, the target trait must be significantly heritable, and this parameter is estimated empirically.

Few genetic studies of oyster shell shape have been performed. Many have focused on interspecies variation. Imai and Sakai (1961) reported differences in depth index among different races of Japanese oysters, which persisted in common

garden experiments, implying significant broad-sense heritability of the trait. Hybrids between Hokkaido and Kumomoto strains scored phenotypically intermediate for shell dimensions, regardless of environment, which also demonstrates genetic control. Wada (1986) conducted selection in the pearl oyster, *Pinctada fucata*, for shell width (the measurement equal to whole shell depth in the Pacific oyster), and shell convexity (which is shell depth relative to the sum of length, width and depth) over two generations and estimated realized heritability for "width" as 0.47, and as 0.35 for convexity. Toro et al. (1995) estimated realized heritabilities for shell length in two year old *Ostrea chilensis* between 0.24 and 0.36, and Toro and Newkirk (1991) estimated realized heritability in shell height in 30 month old *O. chilensis* at 0.34 ± 0.12. Ward et al. (2005) estimated heritabilities in Australian two year old Pacific oysters for length, width and depth as 0.58 ± 0.15, 0.26 ± 0.14, and 0.43 ± 0.15, respectively. Ward et al. (2005) also estimated heritabilities for depth and width indices as 0.49 ± 0.16 and 0.30 ± 0.12. However, they reported low genetic gains for both indices, possibly due to density issues or negative correlations between those allometric traits and oyster weight. Batista et al. (2008) reported interspecific differences in depth relative to length between *C. gigas, C. angulata* and their hybrids, showing a basis for genetic control, and suggesting that creation of hybrids or backcrosses could exploit the genetic variation among the two species for selective breeding.

Selective breeding can make additive genetic change in a trait, given consistent breeding objectives, reasonable measurement accuracy and selection intensity, if the trait is significantly heritable. Heritability is the portion of the

phenotypic variance for a trait that is of an additive genetic nature; it is the ratio of additive genetic variance to phenotypic variance ($h^2=\sigma_A{}^2/\sigma_P{}^2$). Thus, heritability can be estimated by quantifying the degree of resemblance among relatives; regressing the trait means of full-sib offspring families on the means of each pair of parents yields a direct estimate of narrow-sense heritability (Falconer and MacKay 1996, Lynch and Walsh 1998). This method is less affected by common environmental effects than variance component analysis, as it estimates the parameter directly, without multiplication due to coefficient of relationship, which also multiplies estimation error.

In the previous chapter of this dissertation, we found that family mean growth and survival for families reared in the subtidal mixed together were very similar to those same families reared separately (Pearson's *r* for individual weight is 0.817). In this study, we used offspring data collected from mixed-family rearing of 1124 offspring oysters from 40 full-sib oyster families to estimate narrow-sense heritability of shell shape dimensions and indices of depth and width using midparent-offspring regression.

Methods

Overview

Parental and offspring oysters were reared in lantern nets in Yaquina Bay, Oregon for two years. Their shells were measured at harvest, and offspring were assigned to parents using microsatellite molecular markers and assignment software. Mid-parent offspring regression was then performed to estimate narrow-

sense heritability, using statistical methods that were robust to heteroscedastic and clustered data.

Culture

The forty full-sib families used in this study were produced as part of the Molluscan Broodstock Program's Cohort 18. The Molluscan Broodstock Program (MBP) is a Pacific oyster breeding and research program, operated through Oregon State University at the Hatfield Marine Science Center in Newport, Oregon. MBP produces two such cohorts per year, plants them at two field sites, and evaluates families for selection after two years.

Families were reared in separate containers through the larval and juvenile phases. Broodstock oysters were conditioned in 18°C sand-filtered seawater from the Yaquina Bay at the Hatfield Marine Science Center and fed them with a mixture of live microalgae species, cultured on-site, until oysters were in fully reproductive condition (Robinson 1992a, 1992b). Those broodstock were strip-spawned to produce full-sib pair-matings; cultures were individually fertilized and stocked in separate containers at equal densities. Seawater for larval cultures was pumped from the Yaquina Bay at high tide, SPF filtered and treated with calcium montmorillonite according to Matson et al. (2006). Larval batch cultures were reared at 25°C in 30L tanks with aeration in a temperature-controlled room (Langdon et al. 2003) and fed with equal rations of live microalgae (Breese and Malouf 1975). Larvae were induced to metamorphose using an epinephrine solution at 2×10^{-4} M for 1 hour (Coon et al.1986). Successfully metamorphosed postlarvae from each tank were transferred to 15 cm diameter convertible

upwelling silos. Silos were held in a semi-recirculating system that received approximately 20 exchanges per day of 1µm-filtered seawater. As the oysters grew to approximately 1.5mm in shell length, they were transferred to a larger upwelling system with a similar exchange rate, and remained there until reaching approximately 4mm shell length, when they were moved to outdoor tanks at the ambient temperature of the Yaquina Bay.

It was empirically determined in Chapter 3 of this dissertation that mixing families as early veligers orpediveligers produced significant departures from a uniform mixture by the time oysters grew to planting size. Further, it was inferred that mixing at the post-larval stage incurred a substantial risk of creating high variation in family representation, should any problems occur in the nursery. Thus, individuals from each family were mixed together at planting size for this study.

Juveniles were randomly selected from separate-family cultures for planting in the field. Both parents and offspring were stocked in lantern nets at approximately equal densities (50 oysters in each net compartment) in the subtidal of Yaquina Bay. This gear is representative of that used in breeding and commercial growout of Pacific oysters on the U.S. West Coast. For detailed field rearing methods, see Chapter 4 of this dissertation.

Parents were shucked at spawning, and their shells were labeled and measured later. After shucking, a sample of mantle tissue was taken from each oyster used in this study (both parents and offspring) and placed in 95% ethanol for DNA extraction and genotyping. Offspring were individually measured whole, live on a digital scallop measuring board (Scielex, Inc., Australia), and then

shucked. Length was measured as the longest dimension of the shell, from the hinge to the opposite shell lip. Width was measured perpendicular to length (i.e. from one side, looking down on the left valve, to the opposite side of the same valve), and depth was the maximum distance from top to bottom shell (outside of left valve to opposite side of right valve).

Molecular methods and parentage assignment

DNA was extracted using a glass-fiber protocol according to Ivanova et al. (2006). Microsatellite markers were chosen by genotyping parents and analyzing those data using P-LOCI (Matson et al. 2008) to identify the most efficient set of markers for parentage assignment. P-LOCI identified Cg049, Cg108, and CgL10 (Li et al. 2003, Magoulas et al. 1998) as able to assign 95.5% of offspring back to their parental pairs in simulations. Parents and offspring were genotyped at those three microsatellite markers, plus an additional one (Cg197, Li et al. 2003), to be conservative. The fourth marker, Cg197, added an additional 2.5% assignment success in simulations. Polymerase chain reactions (PCR) for the four markers were run separately using primers with four different dyes (ABI G-5 dye set: FAM, NED, VIC, and PET, with LIZ ladder) and their products were coloaded on an ABI 3730XL fragment analyzer.

Parentage was assigned using PAPA software (Duchesne et al. 2002) with sex-differentiated, blocked parent files, using the three microsatellites *Cgi*049, *Cgi*108, and *umdCg*L10. The settings used for modeling genotyping error included a global level of transmission error of 0.05, and a distribution of transmission error over alleles of 6, which was slightly more stringent than in Taris et al. (2005) and

Evans et al. (2008). The three loci enabled assignment with a success rate of better than 95%, as simulated beforehand by P-LOCI.

Data analysis

We regressed mean family offspring trait values on mid-parent trait values according to Falconer and MacKay (1996). Traits included length (L), width (W), and depth (D), and allometric traits included D/L, W/L (Brake et al. 2003), D/W, "Depth Index" (Imai and Sakai 1961). We conducted factorial, between subjects, univariate GLM (ANOVA) analyses to produce least squares estimated means, adjusted for nuisance factors, to use in regressions for heritability estimation. Family and net were random effects, and block was a fixed effect.

The linear relationship between mid-parent and mean offspring values was estimated using PROC REG and PROC GENMOD in SAS statistical software, version 9.2 (SAS Institute, 2009). Diagnostics were performed in SAS and MS Excel. The Kolmogorov-Smirnov test was used to evaluate normality, and we used the SPEC command in SAS was used to test whether the mean and mode of variables were significantly different, which is an indication of skewness. Also, the White test in SAS was used to test for heteroscedasticity.

Linear slope (heritability) was estimated using two methods: weighted least squares (WLS) linear regression using PROC REG, and generalized estimating equations (GEE) using PROC GENMOD, both in SAS 9.2. Estimates of standard errors for the parameters were robust to heteroscedasticity in both WLS and GEE methods. An identity link function was specified in PROC GENMOD (normal distribution, linear parameter estimation). We used the acov option in the WLS

method to calculate standard errors using the asymptotic covariance matrix and account for non-constant variance in the observations. The GEE method uses an empirical standard error estimating procedure to allow for heteroscedasticity while implementing an HC_0 correction to the covariance matrix and standard error estimates, and takes into account the fact that observations, which are grouped according to the subject variable, are related. None of the offspring families shared parents; they were all from separate matings. To be conservative, we applied regression methods for clustered data, since we were attempting to estimate the regression slope with the highest precision. The population was produced from crossing oysters that belonged to eight different full-sib families from one cohort with eight different full-sib families from another cohort, and observations were assigned into 14 different groups according to the family that each sire belonged to. Analyses were run using both sire family and dam family. Quasi-likelihood under the Independence model Criterion (QIC and QICu) were used as GEE fit criteria. The QIC statistic is analogous to the more familiar AIC (Akaike's Information Criterion) statistic used for comparing models fit with likelihood-based methods.

In both WLS and GEE procedures, families were weighted for uneven offspring family size, according to Falconer and MacKay (1996) by the equation:

$$W_n = \frac{(n + nB)}{(1 + nB)}$$

where *W* is the weighting factor and *n* is the number of individuals in the family. B was computed as:

$$B = \frac{\left(t - \frac{1}{2}b^2\right)}{(1-t)}$$

where *b* is the slope of the regression of unweighted values, and *t* is the intraclass correlation for family estimated using PROC VARCOMP in SAS. The number of offspring measured per family varied between 8 and 34. Forty families were measured in all.

Allometric traits were expressed as proportions, which were then arcsine, square root transformed to adhere to normality and homogeneity of variance assumptions of the GLM (Sokal and Rohlf 1995, Zar 1996). All traits were analyzed in the offspring, and least squares means were estimated using PROC GLM in SAS, version 9.2. Family was entered as a random effect, block as a fixed effect, and block by family interaction as a random effect. Appropriate F-statistics were generated using the TEST option in SAS PROC GLM for mixed effects models. Although we have no reason to suspect that the sexes should have different phenotypic variances, offspring were not at a sufficient stage in gonadal development to be reliably sexed; therefore that information could not be incorporated into the heritability estimation.

Results

Mid-parent shell depth was a significant predictor of offspring shell depth when using both WLS regression and GEE ($p=0.0044$ and <0.0001 respectively), and heritability was estimated as 0.4040 in both methods (Tables 1 and 2). The standard error for the estimate was 0.1335 using WLS and 0.0735 using GEE (Table 2). The WLS R^2 was 0.2530 (Table 1, Figure 1), and the fit criteria for GEE

were QIC = 40.5733 and QICu = 42. Model fit is indicated by QIC; it approximates QICu for a correctly specified model. Using dam family as the grouping variable produced the best model fit, while sire family as the grouping variable resulted in the same parameter estimates, but slightly poorer fit and larger standard error (QIC = 41.5684, QICu = 42.000, SE = 0.1410, Table 2.b.). Mid-parent shell width was also a significant predictor of offspring shell width when using both WLS regression and GEE (p=0.0155, and 0.0079, respectively), and heritability was estimated as 0.2827 in both methods (Tables 1 and 2). The standard error for the estimate was 0.1115 using OLS and 0.1025 using GEE (Table 2). The WLS R^2 was 0.1888 (Table 1, Figure 1), and the fit criteria for GEE were QIC = 42.1391, and QICu = 42. Using dam family as the grouping variable produced the best model fit, and sire family as the grouping variable resulted in the same parameter estimates, but slightly poorer fit and larger standard error (QIC = 41.5684, QICu = 42.000, SE = 0.1064, Table 2.b.). Heritability estimates for length (L) and the allometric traits, D/L, and "Depth Index" were not significant (p=0.224, 0.338, and 0.191, respectively).

Distributions of parent and offspring depth and width were not significantly different from normal using the Kolmogorov-Smirnov test ($p>0.05$), as well as the means and modes of the distributions were not significantly different ($p>0.05$). Parental depth and width tested positive for significant heteroscedasticity using the White test in SAS ($p<0.05$), indicating non-constant variance within each variable, which was accounted for in the estimation procedures, as described in the Methods section.

A summary of GLM output detailing the models used to estimate least squares family means is presented in Table 3. There were significant effects of net [$F(11, 1062) = 2.13$, $p=0.0159$] and family [$F(47, 1062) = 2.45$, $p <0.0001$] on shell depth. Block was not a significant factor affecting shell depth [$F(3, 1062) = 1.86$, $p=0.1344$]. There were also significant effects of net [$F(11, 1062) = 4.53$, $p <0.0001$] and family [$F(47, 1062) = 3.01$, $p <0.0001$] on shell width. Block was also not a significant factor affecting shell width [$F(3, 1062) = 1.86$, $p=0.0649$]. Shell depth was a significant factor in the ANOVA for the WLS regression [$F(1, 38) = 12.87$, $p =0.0009$]. The same was true of shell width [$F(1, 38) = 8.84$, $p =0.0051$].

Discussion

Results from this study confirm that shell depth and width are under a high degree of additive genetic control in *C. gigas*. Our heritability estimates for these two traits are nearly equal to the two site averages of Ward et al. (2005) for depth and width in a Pacific oyster breeding program in Australia in which families were reared separately, which supports the conclusion of the previous chapter of this dissertation that rearing Pacific oysters in Mixed and Separate family conditions yields very similar results. These results also suggest that breeders can expect to make significant change for these two characters using selective breeding, given consistent breeding objectives, reasonable measurement accuracy and selection intensity. However, shell length, or indices of depth or width relative to length were not heritable, which is not encouraging for selective breeding on these characteristics in the commercial densities used in this study.

Density is likely an important factor influencing shell shape and its heritability. Ward et al. (2005) reported successfully estimating heritability of depth and width ratio traits after lowering densities beneath standard commercial levels. Shell shape in this oyster is highly plastic (Quayle 1988), and length, the primary vector of growth, may be the most plastic of the three basic dimensions describing shell shape. Periodic deliberate "pruning" of shell length (discussed earlier) produces much shorter oysters, which are then deeper and wider, relative to length. Oysters growing in a fixed position, crowded either by other oysters, or immersed in the mud, become very long and narrow. Given this plasticity, oyster density in the growing compartment can be expected to affect heritability of length and associated traits. Other traits including growth, reproductive effort and resource allocation have been determined to be plastic in the Pacific oyster as well (Ernande et al. 2004), fitting the species life history, growing in a highly variable environment

Although Ward et al. (2005) found oyster weight to be positively phenotypically correlated with depth and width indices, weight was negatively genetically correlated with the same indices. At the same time, Ward et al. (2005) reported that weight was positively correlated with individual shell dimensions, both phenotypically and genetically. Those results have important implications; they highlight the importance of environmental effects on shell shape, and suggest that common environmental effects were influential for these allometric shell traits in the variance component estimation from Ward et al. (2005). Our study design was not prone to common environmental effects, as the offspring from different

families were reared mixed, which avoids confounding shell shape with density dependent growth as a result of family specific survival. Offspring density varied from the initially equal stocking density independently from family-specific survival.

Use of the formula given in Lynch and Walsh (1998) for predicting the standard error of an heritability estimate for 40 families, using heritability values of 0.40, and 0.28 (as used in our study for depth and width), predicts standard errors of 0.218 and 0.208, which suggests that our number of families (48) should be sufficient for detecting heritability of depth index using parent-offspring regression, given the true value is close to 0.49 as estimated by Ward et al. (2005). This suggests that statistical power is sufficient in our study.

Reducing densities below commercial levels may indeed reveal heritability of length and allometric traits such as depth index, but production systems would also need to change in order to realize the phenotypic result of breeding in a reduced density environment. Production costs per growing unit would be higher at lower density, and would therefore need to be balanced by the higher market price of selectively bred, shapely oysters for breeding in these conditions to be economically viable.

Further efforts at standardizing shell depth and width will need to be undertaken, in order to investigate the heritability of shell shape independent to overall growth.

An academically interesting future topic of investigation would be the heritability of shell shape plasticity, and its inverse, canalization. Characterized by

growing in a highly variable environment, plasticity is integral to the evolution of the Pacific oyster. Genetic parameters of plasticity are rarely investigated, especially in commercially important species.

Conclusion

In this study, we estimated heritability of shell depth as 0.404 ± 0.14 and of shell width as 0.287 ± 0.11, which are very similar to estimates from other populations of this species in Australia, estimated using variance components, although length, D/L and depth index, were found not heritable. This confirms that sufficient additive genetic variation exists for depth and width to make progress using selective breeding for these traits in this population, although selective breeding should not necessarily supplant physical methods in the commercial environment. Reduction of oyster rearing density below current commercial levels may create an environment in which length and ratios of length to other shell dimensions are heritable, but breeding would only be useful if trait values can be realized in production systems.

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Table 5.1. Regression (WLS) coefficients and statistics for weighted, mid-parent-offspring regression of shell depth (D) and width (W).

Variable	R^2	Adj. R^2	Parameter Estimate	SE	t	Pr > t	n
Intercept	-	-	43.5239	7.9160	5.50	<0.0001	40
Width	0.1888	0.1674	**0.2827**	0.1115	2.54	**0.0155**	40
Intercept	-	-	20.9414	5.4397	3.85	0.0004	40
Depth	0.2530	0.2333	**0.4040**	0.1335	3.03	**0.0044**	40

Table 5.2. Generalized linear model parameter estimates and statistics computed using GEE for weighted, mid-parent-offspring regression of shell depth (D) and width (W) in millimeters.

a. Subject effect = dam family.

Variable	QIC	QICu	Parameter Estimate	SE	95% CI		Z	Pr > Z	n
Int.	-	-	43.5239	7.1498	29.5104	57.5373	6.09	<0.0001	40
Width	42.1391	42	**0.2827**	0.1025	0.0818	0.4836	2.76	**0.0058**	40
Int.	-	-	20.9414	3.1416	14.7840	27.0988	6.67	<0.0001	40
Depth	40.5733	42	**0.4040**	0.0755	0.2561	0.5519	5.35	**<0.0001**	40

b. Subject effect = sire family.

Variable	QIC	QICu	Parameter Estimate	SE	95% CI		Z	Pr > Z	n
Int.	-	-	43.5239	7.5366	28.7524	58.2953	5.78	<0.0001	40
Width	41.5684	42	**0.2827**	0.1064	0.0741	0.4913	2.54	**0.0079**	40
Int.	-	-	20.9414	5.5159	10.1306	31.7523	3.80	0.0001	40
Depth	43.5803	42	**0.4040**	0.1410	0.1410	0.6671	3.01	**0.0026**	40

Table 5.3. Summary of general linear model (GLM) output for offspring shell depth and shell width.

Trait	Factor	F	df-n	df-d	p
Depth	model	3.41	61	1062	<0.0001
	block	1.86	3	1062	0.1344
	net	2.13	11	1062	0.0159
	family	2.45	47	1062	<0.0001
Width	model	3.41	61	1062	<0.0001
	block	2.42	3	1062	0.0649
	net	4.53	11	1062	<0.0001
	family	3.01	47	1062	<0.0001

Table 5.4. Regression ANOVA output for shell depth and shell width.

Trait	Factor	F	df-n	df-d	p
Depth	model	12.87	1	38	0.0009
Width	model	8.84	1	38	0.0051

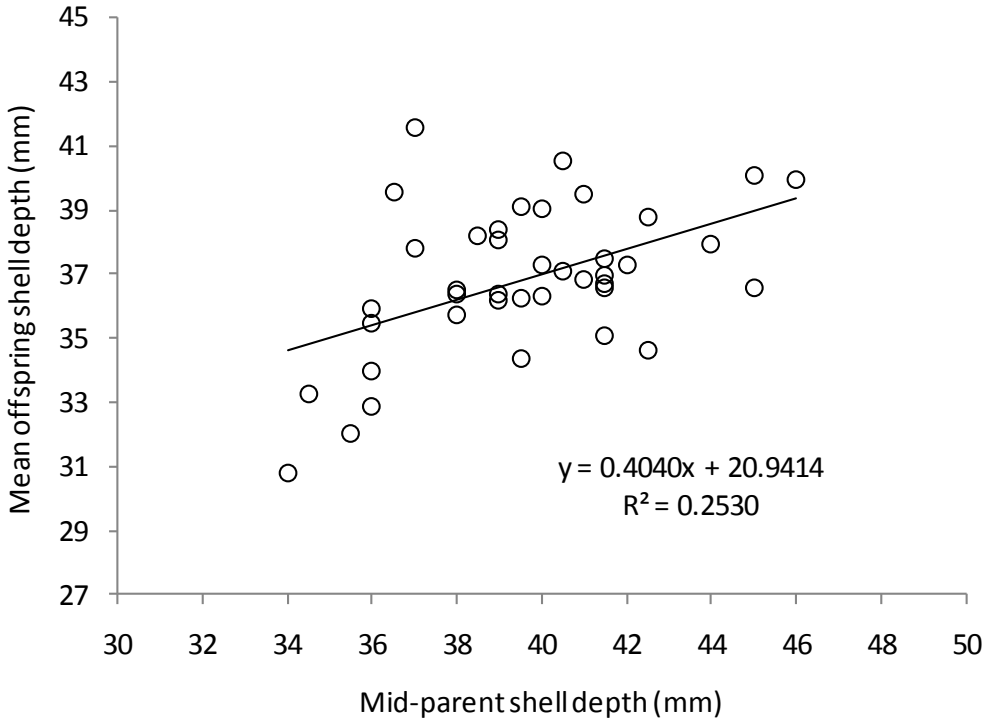

Figure 5.1. Regression of mid-parent on offspring for shell depth. Mid-parent values are displayed on the x-axis, and mean values of offspring on the y-axis.

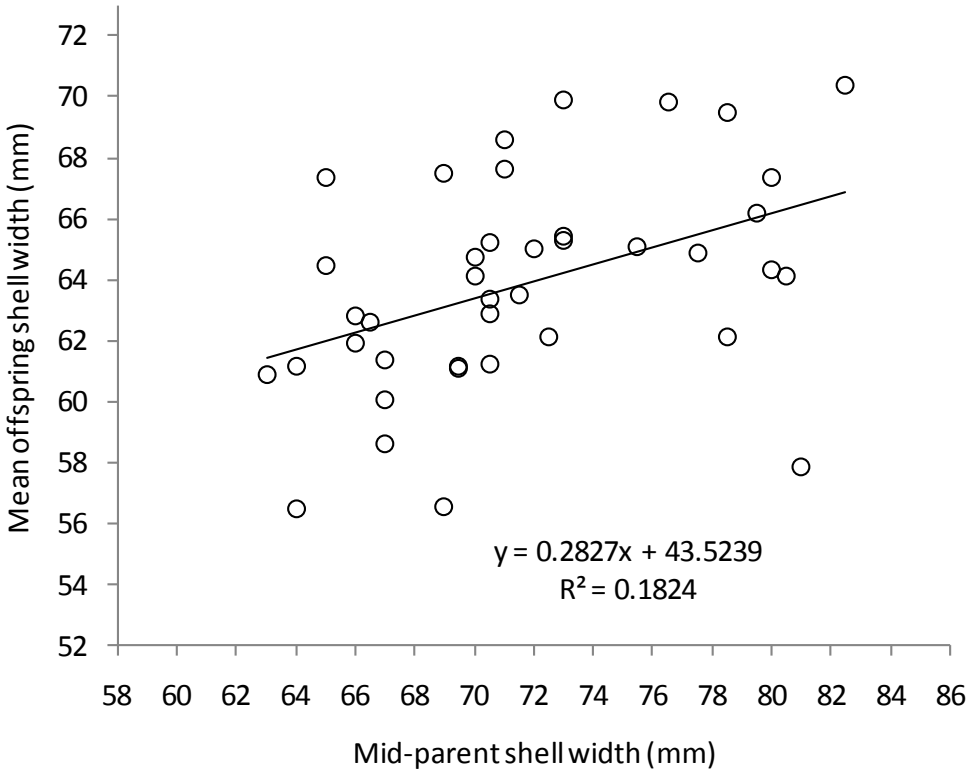

Figure 5.2. Regression of mid-parent on offspring for shell width. Mid-parent values are displayed on the x-axis, and mean values of offspring on the y-axis.

CHAPTER 6. CONCLUSIONS

The aim of this dissertation was to determine the suitability of mixed-family selection (MFS) for breeding Pacific oysters using marker-based pedigree reconstruction and test efficacy of MFS relative to more traditional methods. This research addressed three important, but unanswered questions concerning MFS: 1) What is the most cost-efficient suite of genetic markers that can be used for reconstructing Pacific oyster pedigrees? 2) At what point in the life cycle can we mix families in equal numbers and expect them to still be equally represented when they are planted in the field? 3) What is the optimal strategy of selectively genotyping individuals to implement mixed-family selection, and does it compare favorably with traditional separate-family selection?

To address the first question, we developed novel computer software, called P-LOCI, which identifies the most efficient set of codominant markers for assigning parentage at a user-defined level of success, using either simulated or actual offspring genotypes of known parentage. Simulations can incorporate linkage among markers, mating design, and frequencies of null alleles and/or genotyping errors. We tested P-LOCI with actual and simulated microsatellite and SNP data, varying levels of polymorphism, distribution of alleles among parents, number of parents, mating design complexity, degree of linkage among markers, and locus-specific frequencies of null alleles and genotyping errors. It performed as expected in terms of reflecting the relationships between the variables and predictions of numbers of markers necessary to assign parentage. An interesting

outcome was that the top individually ranked loci do not necessarily constitute the most efficient group of loci for assignment, and that group is not necessarily the best for all populations. The most efficient group of loci can be comprised of both top individually-ranked and middle or lower individually-ranked loci, something that cannot be readily predicted from other currently available software, or individual measures of marker assignment power. This software is unique in this respect, as well as in its ability to incorporate marker linkage. It fills a needed niche, as a flexible, easy to use and powerful tool for conducting efficient parentage analysis. P-LOCI is available for Windows systems at http://marineresearch.oregonstate.edu/genetics/PLOCI.html

To address the second question, we conducted larval and nursery experiments that clearly showed that variance in family representation within mixtures increased with the amount of time elapsed since mixing occurred. Family representation within mixes was no longer equal at 48 days post-fertilization (planting size) in groups that were mixed at 24 hours (straight-hinge larvae) and 13 days (pediveliger larvae), while groups that were mixed at 27 days (post-larvae) remained uniformly mixed. While post-larval mortalities in this experiment were negligible, periodic mortalities should be expected at any stage of production, and could easily lead to high variability in family representation at the PL stage as well. For these reasons, and others mentioned earlier, planting size is the most prudent time to mix families for MFS. Mixing families at stages earlier than this for selection on field traits would require pre-planting genotyping of large samples

for estimation of initial family representation, which would add substantial cost, or other special considerations.

In addressing the third question, we found that rearing oysters of different families mixed together for two years in the field yielded very similar results to rearing them separately, when comparing family mean individual weight and survival (r = 0.817 for two-site average of individual weight at harvest). This demonstrates that it is unlikely that associative effects are of great importance in the Pacific oyster at this range of stocking densities. Differences in how the two methods deal with environmental variation, and estimate family means due to their experimental design, together with sample size differences (especially important for survival estimation), and high within-plot environmental heterogeneity at the intertidal site, could easily account for the remaining discrepancy in the correlation of family means between methods. The mixed method was well-suited for selection by individual traits by family or walk-back selection, but would incur high laboratory costs in order to estimate survival with an acceptable level of precision and accuracy. Assuming 50% survival, we estimated that a mixed-family approach intended for selecting on survival or yield would cost approximately 19% more than conducting the same selection by rearing families separately, and the mixed approach would still make a large sacrifice in precision and accuracy of survival estimation. However, assuming 69% survival, which was the two-site average for this study, the cost of mixed-family approach jumps by 18%, to 41% more than the separate method. Survival has a significant effect on the cost of the mixed method for estimating survival or yield, increasing the number of oysters

that must be genotyped, but no effect on the cost of the separate-family or walk-back methods, according to the designs used in this study. The strength of the mixed approach lies in selecting on individual traits; using a mixed approach to select solely on individual weight could be done using walk-back selection for approximately 70% of the cost of a typical separate-family planting.

Finally, we estimated heritability of shell depth as 0.404 ± 0.14 and of shell width as 0.287 ± 0.11, which are very similar to estimates from other populations of this species in Australia, estimated using variance components, although length, D/L, W/L and depth index, were found not heritable. This confirms that sufficient additive genetic variation exists for depth and width to make progress using selective breeding for these traits in this population, although selective breeding should not necessarily supplant physical methods currently in use in the commercial environment. Reduction of oyster rearing density below current commercial levels may create an environment in which length and ratios of length to other shell dimensions are heritable, but breeding would only be useful if trait values can be realized in production systems.

Taken together, we found that mixed-family rearing is a viable option for Pacific oyster breeding, given some important restrictions. For selecting on field traits, juveniles should be mixed at planting time, due to family-specific variation in larval and nursery survival, otherwise genotyping costs would become prohibitive. Also, reliable estimation of aggregately measured trait values is currently significantly more expensive using mixed-family rearing than for families reared separately. The difference in cost lies in genotyping, and although

laboratory costs of genotyping are dropping, labor for non-destructive tissue sampling, tagging and maintaining large numbers of animals from harvest until selection is also a substantial part of the overall cost, and skilled labor costs are not dropping. However, walk-back selection should be an economically viable strategy for breeding for individually measured traits, such as individual weight, shell shape, or shell color, as long as quantifying survival is not important to the breeder.

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APPENDICES

APPENDIX A. USER MANUAL FOR P-LOCI VERSION 1.0 PARENTAL ASSIGNMENT LOCI CHOICE SOFTWARE

Program overview

P-LOCI determines the most efficient set of codominant marker loci for assigning parentage to a given set of potential diploid parents. It was designed primarily to work with microsatellites, but works with any codominant loci in diploid organisms, although the genotyping error simulation feature is available for microsatellite data only. The program can simulate offspring genotype data utilizing mating design, marker linkage, locus-specific frequencies of null alleles and genotyping error. It can then use simulated offspring genotypes or actual genotypic data from subset of offspring with known parentage to rank marker loci by their discriminatory power, and choose the most efficient suite of loci to obtain a user-defined level of assignment success. P-LOCI was written in C++ and developed to run in Microsoft Windows XP.

This program is provided "as-is". The authors and providers give no warranties, expressed or implied, regarding the performance of this software. You may distribute this program freely, so long as the following conditions are met: the program remains intact without modification, the user manual file is included without modification, no fee of any kind is charged.

How to run P-LOCI

To run P-LOCI, simply enter your input files (a minimum of parent and mating files) into the appropriate boxes by using the dropdown menu or browse options. Click “resample”. P-LOCI will prompt you to enter the number of offspring datasets you wish to simulate; enter the number. Be aware that the program produces a new offspring file, ranks loci and determines the best locus

sets for each of the requested datasets, and this increases computing time proportionately. Computing time also depends on the number of families produced, the number of offspring per family, whether error is simulated and whether a linkage file is used. When the simulated offspring file is produced, inspect or save it if you wish. When the user chooses to produce one offspring dataset, P-LOCI will produce a file that the user can save. Multiple offspring datasets are temporarily held in memory and are not retrievable for saving or printing. Finally, click "execute". P-LOCI will then determine the most discriminating set of loci for assigning parentage using your parental population and mating design, and the results will open in another window as a report file that you can save.

Options on the interface

There are six additional options on the interface with which the user can customize the conditions of the analysis. Figure A.1 shows a screenshot of the interface with explanatory callouts for important features. First, one can choose which loci to include from the dropdown list; not all the loci in the parental file need to be considered. Second, the user can change the minimum percent of the offspring that are required to be successfully assigned to parents before the program stops, by entering a value into the box. Third, one has the option of allowing a mismatching genotype at one or more loci for each offspring. This option can be useful when there are typing errors or null alleles present in a dataset. Fourth, the conservative user can have P-LOCI determine the best locus combination with one more locus than is necessary to achieve the desired

assignment success rate. Fifth, the code for null allele can be specified by the user. This is primarily to allow for the use of two, four, or six digit data with the null allele simulation option. Last, the user should choose microsatellite or SNP data. Choosing “microsatellite” will enable the genotyping error simulation option, which is not valid for SNP data.

Input files

P-LOCI will accept four types of input files, including parental, mating, linkage, and actual offspring files. The software needs a minimum of two files to run, a parental file and a mating file. The parental file includes the genotypes of all parents at all marker loci and the names of the parents, plus error information, in a modified genepop format (Table A.1). If the user does not have a pre-conceived mating design, they should enter an all-combinations mating file produced using other software, such as Excel.

The mating file consists of the names of the parents arranged in mating pairs, along with the number of offspring to generate for each cross (Table A.2). The user can enter any arrangement of parents they wish, including the same parents multiple times in different arrangements, such as in a full-sib/half-sib breeding design. All parental genotypes that are present in the parental file do not need to be used in the mating file. Females are entered first.

The user may also wish to enter a linkage file, which contains the linkage map for their species or population. Intermarker recombination frequencies are calculated from the file, which are then used to regulate recombination in producing the multilocus simulated offspring genotypes. Without a linkage file,

alleles are chosen randomly from each parent at each locus to produce the simulated offspring. The linkage file contains six columns of data including the gender, linkage group, marker name, marker distance from one end of the linkage group, and intermarker distances. Map distance can be entered using either the Kosambi or Haldane function (Liu 1997, Lynch and Walsh 1998), and must be denoted in the top row. The format for the linkage file is illustrated in Table A.3.

The last type of input file is the actual offspring genotype file. The format for this file is exactly the same as the simulated offspring file that P-LOCI generates using parental genotypes (Table A.4). The difference is that the user enters actual offspring genotypes and corresponding mating information in place of the simulated data. It may be useful for the user to create such a file when a linkage map, estimates of null allele frequency and genotyping error rates are not available. This information is useful for realistic offspring genotype simulation. However, genotypes from actual offspring inherently include the effects of null alleles, genotyping error and marker linkage. Thus genotyping a *small number* of offspring of known parentage (e.g. from offspring of experimentally controlled crosses or observed matings) may still provide offspring data from which to make a realistic and economical determination of the best set of markers for parentage assignment in a data-poor situation.

Null allele and genotyping error

P-LOCI can incorporate two types of common errors into the simulated offspring genotypes: segregating null alleles, and random genotyping/mutation errors. The user can enter locus-specific frequencies of null alleles and rates of

genotyping/mutation error into the parental file. Locus-specific expected null allele frequencies can be estimated in a number of ways (e.g. Kalinowski and Taper 2007, Van Oosterhout et al. 2004), and must be computed by the user a priori. The simulated multilocus offspring genotypes created will reflect these errors accordingly, and the software will produce a new, modified parental file including errors at the user-specified frequencies, which the user can review. The genotype/mutation error option is only intended for alteration of fragment sizes using microsatellite markers.

Error module input

Locus-specific error rates are input into the parental file, following each locus name in a row (instead of a column, as in the previous parent file example), in the following order, space-delimited: locus name, expected frequency of null alleles, frequency of genotyping/mutation error, number of bases for genotyping/mutation error (Table A.5).

Output files

When P-LOCI produces output files, they appear in the same folder as the parental input file that the user chooses. P-LOCI produces four types of output files: a simulated offspring file, a loci report file, a modified parental file, and a linkage report file. The simulated offspring file lists the name of the parent file and the linkage file (if any) used to generate the offspring, the names of the parents used to generate each offspring, and the genotype of each offspring at each marker locus (Table A.4).

Single dataset output

The loci report file gives the paths of the files used in the run, followed by a list of the loci used and corresponding user-defined error rates for each, after which appears another list of all the loci used with their assignment success scores, in order from highest to lowest. After that, the report lists the best pair, followed by the best triplet, and so on. Next to the best single locus and all of the best locus combinations, P-LOCI lists the percentage correctly assigned as well as the percentage incorrectly assigned. When those two figures sum to less than 100%, the remaining offspring have ambiguous assignment results (Table A.6).

Multiple dataset output

When multiple simulated offspring datasets are produced, P-LOCI outputs single locus ranks and best locus sets for each dataset, plus gives a summary at the end of the output file (Table A.7). This summary gives the number of times and the percent of time that each locus appeared in the best set of loci, the number of times that each locus achieved a particular ranking, and average individual locus rankings over all offspring datasets.

Linkage detail file

The linkage detail file gives details of the offspring simulation routine when the user inputs a linkage map. The file is arranged so that one row corresponds to the details of one simulated offspring. The columns in order from left to right in the linkage detail file are (Table A.8):

ID: identification of individual offspring by cross (e.g. female five by male six, for individual number one in line one of Table A.8).

Locus: gives the loci in order entered in the parental file by name.

Group: the linkage group of the current locus interval being considered.

Sex: gender of the current parent under consideration.

Distance: gives the distance across the marker interval from the current locus to the previous locus. This will read "-1.0" in the first position on each linkage group, signifying "not applicable" since there is no locus before the first one.

Odds: gives the probability that a crossover will occur in the current marker interval under consideration. This will read "0.0" in the first position on each linkage group, signifying "not applicable", since a crossover cannot occur between a locus on one linkage group and a locus on another linkage group by definition.

Random: gives a random number for comparison with the recombination frequency. If the random number is smaller than the recombination frequency, a crossover occurs.

Flip: indicates whether or not a crossover occurred.

Phase: indicates the phase of the current allele, i.e. chromatid one or two.

Genotype: lists the two parental alleles that the offspring allele will be chosen from, in order of phase. The first allele in the parental genotype is phase one, the second, two.

Allele: indicates the allele that P-LOCI chose for the offspring from the current parent, given linkage information.

Modified parental file

When the user selects a parental file using the software interface, P-LOCI produces a modified parental file that contains the errors that the user specified. It will have the same name as the original parental file with the added words "with simulated errors" at the end. It will include the code the user specifies before initiating the software, such as "999" wherever a null allele has been inserted, and genotype/mutation errors at the frequencies specified by the user in the parental file (addition or subtraction of a given number of base pairs).

Sample files

We have provided a few very simple sample input files to enable users to familiarize themselves with P-LOCI and explore it before creating their own files. Parent file names begin with "PAR", mating files begin with "MAT" and the linkage map file name starts with "LINK". You can use these simple files as-is or modify them to see how the results reflect differences among markers in number of alleles, distribution, null allele frequency, typing error frequency and marker linkage, as well as how the results are affected by different mating designs.

In some parental files the markers vary in their number of alleles, and others by their distribution. Two mating files are included, one with full sibs, and another with both full and half sibs.

You can use the parental file “PAR_Toy_10alleles_3loci_allsame” together with the mating file “MAT_Toy_halfsib10pr_100offeach” several times with and several times without the linkage file “LINK_Toy_map” to illustrate what happens using three independently assorting markers (unlinked) versus the same three markers when two of them are tightly linked. Linkage has the most obvious effect on the results when all other factors are equal.

References

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Table A.1. Parental file format. The title name, if any appears in the first row. Marker names appear in the first column, followed by the word “pop” to separate them from individual parent names, including gender as “M” for male or “F” for female. Parental genotypes appear in subsequent columns, in order of marker name, from column one.

```
Title line "Sample Parent Genotypes"

CgXX1
CgXX2
CgXX3
pop
2500F ,     116116      105105      102122
2501M ,     149156      105107      120120
2502F ,     128137      099099      083109
2503M ,     122141      103109      103111
2504F ,     132132      105105      085124
2505M ,     148157      103103      106111
2506F ,     128168      103107      109113
2507M ,     126126      103103      125145
2508F ,     122124      099099      102124
2509M ,     137158      103105      143143
```

Table A.2. Mating file format. Mating pairs are listed by rows, female first, and the desired number of offspring to be produced is listed in the right-hand column.

2500F X	2501M =	100
2502F X	2503M =	100
2504F X	2505M =	100
2506F X	2507M =	100
2508F X	2509M =	100

Table A.3. Linkage map input file format. From left to right, column one is the gender of the map, two is linkage group number, three is marker name, four is map distance from the end of the linkage group, five is the marker order and six is intermarker map distance. In the top row, the word “Kosambi” can be changed to “Haldane” to reflect one of the two map distance functions.

```
<mapunits>Kosambi</mapunits><mapdistances>0,i</mapdist
ances>
f     1     CgXXX 0     1     0.0
f     1     CgXXX 16.9  2     16.9
f     1     CgXXX 24.9  3     8.0
f     1     CgXXX 28.1  4     3.2
f     1     CgXXX 28.7  5     0.6
f     1     CgXXX 28.7  6     0.0
f     1     CgXXX 40.7  7     12.0
f     1     CgXXX 41.1  8     0.4
f     1     CgXXX 41.1  9     0.0
f     1     CgXXX 42.1  10    1.0
f     1     CgXXX 42.1  11    0.0
f     1     CgXXX 42.5  12    0.4
f     1     CgXXX 58.9  13    16.4
f     1     CgXXX 78.1  14    19.2
f     1     CgXXX 102.2 15    24.1
f     1     CgXXX 119.4 16    17.2
f     1     CgXXX 127.9 17    8.5
f     1     CgXXX 137.4 18    9.5
f     1     CgXXX 141.5 19    4.1
f     1     CgXXX 147.9 20    6.4
f     2     CgXXX 0     1     0.0
f     2     CgXXX 3.1   2     3.1
f     2     CgXXX 7.1   3     4.0
f     2     CgXXX 9.5   4     2.4
f     2     CgXXX 10.9  5     1.4
f     2     CgXXX 13.9  6     3.0
f     2     CgXXX 19.8  7     5.9
f     2     CgXXX 32.3  8     12.5
f     2     CgXXX 60.8  9     28.5
f     2     CgXXX 67.2  10    6.4
f     2     CgXXX 108.3 11    41.1
</linkage>
```

Table A.4. Example of an offspring file, either simulated or user-written with actual data. The left-hand column delineates the parents of each offspring, and genotypes appear in the columns to the right, in the same order as the loci are listed in the title line. In this case, the name of the parental file from which the simulated offspring were generated is given in the top row.

C13_parental_file_ploci.txt'
"CgXX1, CgXX2, CgXX3"
Pop

"2500FX2501M-1,"	116149	105107	102120
"2500FX2501M-2,"	116156	105107	122120
"2500FX2501M-3,"	116149	105105	102120
"2500FX2501M-4,"	116149	105107	102120
"2500FX2501M-5,"	116156	105105	122120
"2500FX2501M-6,"	116149	105105	122120
"2500FX2501M-7,"	116156	105107	122120
"2500FX2501M-8,"	116149	105105	102120
"2500FX2501M-9,"	116156	105105	122120
"2500FX2501M-10,"	116149	105105	122120

Table A.5. Parental file with error rates specified. Locus “CgXX1” has a user-defined null allele frequency of 11%, a genotyping error rate of 2% and a genotyping error size of plus/minus one base pair. Null alleles will be incorporated into parental homozygotes or parents coded by the user as null (null allele = 999). Genotyping error will be incorporated into the simulated offspring file.

```
"Title line""Toy parents 3 loci, 10 alleles"

CgXX1<0.11 0.02 1>, CgXX2<0.0 0.0 0>, CgXX3<0.0 0.0 0>

pop
2500F   ,       116116  105105  102122
2501M   ,       149156  105107  120120
2502F   ,       128137  099099  083109
2503M   ,       122141  103109  103111
2504F   ,       132132  105105  085124
2505M   ,       148157  103103  106111
2506F   ,       128168  103107  109113
2507M   ,       126126  103103  125145
2508F   ,       122124  099099  102124
2509M   ,       137158  103105  143143
```

Table A.6. P-LOCI report output file (next page), showing in order from top to bottom, the path and filename of the input files used, error rates by locus as specified by the user, number of mismatches allowed, user-specified assignment accuracy as a percentage and number of offspring, single locus rankings by assignment success, and best locus sets. This report was produced using one simulated offspring dataset. If it had been produced using multiple datasets, this data would be repeated in the report for each offspring dataset, followed by a summary report of average rankings and number of times each locus appeared in the best locus set.

```
P-LOCI Output

Parents File = C:\path\filename.txt
Mating History File = C:\path\filename.txt
Linkage Map = C:\path\filename.txt

N rate = null allele rate, T rate = typing error rate, T
size = typing error size
Locus  N rate T rate T size
CgXXX  0.000000     0.000000     1
CgXXX  0.000000     0.000000     1
CgXXX  0.000000     0.000000     1
.
.
.
CgXXX  0.000000     0.000000     1

Allowing 1 incompatible locus during assignment

User-specified Assignment Accuracy = 95
Minimum Correctly Assigned (for specified accuracy) = 1026
out of 1080

Rank 1 = CgXXX      score = 66.296295
Rank 2 = CgXXX      score = 59.259258
Rank 3 = CgXXX      score = 51.944443
Rank 4 = CgXXX      score = 43.796295
Rank 5 = CgXXX      score = 39.722221
Rank 6 = CgXXX      score = 37.500000
Rank 7 = CgXXX      score = 36.296295
Rank 8 = CgXXX      score = 34.166668
Rank 9 = CgXXX      score = 27.685184
Rank 10 = CgXXX     score = 13.888889
Rank 11 = CgXXX     score = 13.333333
Rank 12 = CgXXX     score = 4.629630
Rank 13 = CgXXX     score = 4.259259
Rank 14 = CgXXX     score = 0.000000
Rank 15 = CgXXX     score = 0.000000

Using 1 locus, best locus:
CgXXX  Correctly Assigned 66.2963%
       Incorrectly Assigned 0%

Using 2 loci, best locus set:
CgXXX, CgXXX
       Correctly Assigned 90.2778%
       Incorrectly Assigned 0%

Using 3 loci, best locus set:
CgXXX, CgXXX, CgXXX
       Correctly Assigned 94.1667%
       Incorrectly Assigned 0%

Using 4 loci, best locus set:
CgXXX, CgXXX, CgXXX, CgXXX
       Correctly Assigned 95.0926%
       Incorrectly Assigned 0%

Used 4 loci to meet accuracy specifications as requested.
```

Table A.7. Multiple dataset summary output, listing the number of times and the percent of time that each locus appeared in the *best set* of loci and the number of times that each locus achieved a particular *individual ranking*. In this simple example, locus B was ranked first in three of three datasets, locus A was ranked second in three of three datasets, and locus C was ranked third in three of three datasets. Average ranks over all offspring datasets are also given.

Loci ranked for every dataset

Locus	Rankings	Times in Best Set	% in Best Set	1	2	3
A		3.000	100.000	0	3	0
B		3.000	100.000	3	0	0
C		0.000	0.000	0	0	3

Locus performance, averaged over 3 datasets

Locus	Score	Rank
A	53.156	2.000
B	62.444	1.000
C	21.956	3.000

Table A.8. Linkage detail file format, giving the details of simulated offspring production when the user enters a linkage map. Information given by this file includes offspring identification, current locus, linkage group, gender of the current parent, intermarker distance, recombination frequency, a random number, whether or not a crossover occurred, the two parental alleles under consideration, and the allele chosen for the offspring from the current parent.

ID	Locus	Group	Sex	Distance	Odds	Random	Flip	Phase	Genotype	Allele
5FX6M-1,	1 (A)	1	f	-1	0	0.757775		2	9,2	2
5FX6M-1,	2 (B)	1	f	0.072	0.071506	0.93292		2	9,2	2
5FX6M-1,	3 (C)	2	f	-1	0	0.740989		2	9,2	2
5FX6M-1,	1 (A)	1	m	-1	0	0.521073		2	3,7	7
5FX6M-1,	2 (B)	1	m	0.072	0.071506	0.053652	yes	1	3,7	3
5FX6M-1,	3 (C)	2	m	-1	0	0.385144		1	3,7	3

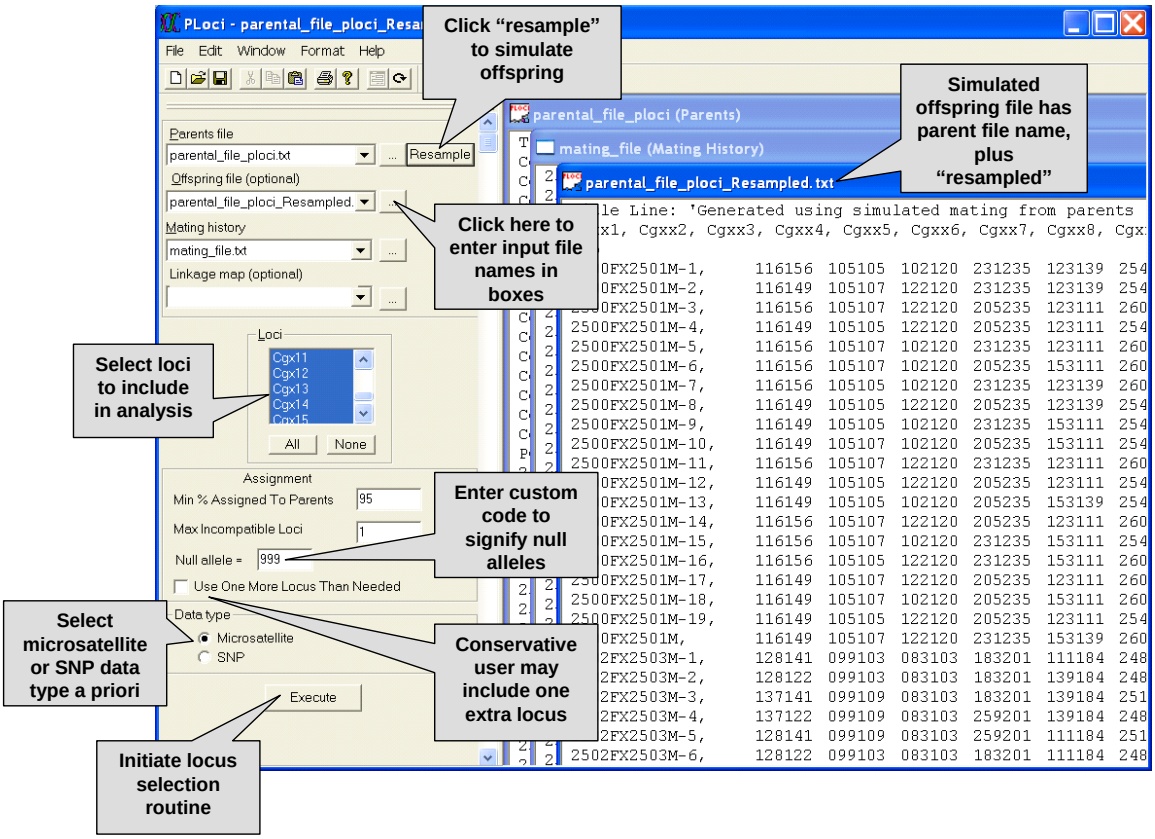

Figure A.1. Screenshot of P-LOCI software interface, showing where to enter input files and other important operating information.

APPENDIX B.

Table B.1. Least squares estimated means for individual weight in the mixed and separate treatments, at the subtidal and intertidal sites, sorted by family. P-values for all estimates are <0.0001.

Subtidal individual weight						Intertidal individual weight					
Mixed			Separate			Mixed			Separate		
family	indwtg	SE	family	indwtg	SE	family	indwtg	SE	family	indwtg	SE
1	137.746	9.336	1	121.382	4.726	1	78.957	5.501	1	79.392	3.397
2	123.493	10.843	2	110.693	4.418	2	78.702	8.103	2	70.779	3.397
3	121.89	10.231	3	120.667	4.418	3	88.444	5.979	3	79.295	3.397
4	137.186	12.269	4	125.633	4.418	4	95.201	5.609	4	75.89	3.397
5	107.57	8.227	5	112.591	5.106	5	60.602	5.399	5	66.612	3.397
6	117.704	8.499	6	120.957	4.418	6	89.184	5.504	6	71.038	3.397
8	130.54	8.364	8	126.088	4.418	8	90.836	5.402	8	77.96	3.397
9	89.354	9.19	9	102.825	4.418	9	57.333	6.272	9	58.622	3.397
10	110.495	11.852	10	123.361	4.418	10	78.826	6.443	10	76.823	3.397
11	114.754	8.821	11	113.415	4.418	11	67.432	5.981	11	76.355	3.397
12	124.86	9.169	12	131.245	4.418	12	92.605	5.615	12	79.254	3.397
13	156.877	16.233	13	133.271	4.418	13	74.834	9.919	13	76.074	3.397
14	100.593	10.223	14	111.155	4.418	14	73.841	5.609	14	76.51	3.397
15	137.718	8.813	15	126.312	4.418	15	100.626	5.85	15	82.298	3.397
16	141.181	9.75	16	125.665	4.418	16	85.749	5.609	16	76.77	3.397
17	98.085	11.455	17	117.444	4.418	17	68.079	5.501	17	68.603	3.397
18	122.378	8.958	18	133.001	5.106	18	77.436	5.4	18	78.239	3.397
19	132.435	8.661	19	129.842	4.418	19	86.116	5.979	19	74.389	3.397
20	139.146	9.146	20	118.228	4.418	20	89.044	6.123	20	77.935	3.397
21	112.279	8.664	21	120.615	4.418	21	67.388	5.3	21	65.24	3.397
22	142.809	10.008	22	126.241	4.418	22	93.357	5.725	22	75.751	3.397
23	115.476	9.56	23	106.652	4.418	23	78.882	5.98	23	70.901	3.397
24	125.493	9.367	24	110.148	4.726	24	84.319	6.808	24	76.148	3.397
25	127.273	8.348	25	136.453	4.418	25	80.606	5.504	25	75.658	3.397
26	129.325	11.454	26	118.457	4.418	26	60.193	6.612	26	76.691	3.397
28	123.01	10.246	28	114.254	4.726	28	67.023	5.504	28	79.742	3.397
29	120.592	10.788	29	104.246	4.418	29	76.323	5.98	29	68.109	3.397
32	113.081	8.974	32	114.635	4.418	32	89.776	5.609	32	78.866	3.397
33	90.597	8.66	33	80.087	4.418	33	48.531	6.272	33	58.633	3.397
34	91.584	8.514	34	97.325	4.418	34	57.638	5.729	34	56.94	3.397
35	138.641	9.542	35	127.39	4.418	35	96.944	6.12	35	76.301	3.397
36	109.746	8.496	36	107.749	4.418	36	72.863	5.501	36	76.12	3.397
37	145.245	10.494	37	130.283	4.418	37	86.59	5.728	37	76.623	3.397
39	139.689	8.507	39	117.842	4.726	39	91.082	5.855	39	80.396	3.397
40	137.813	9.547	40	118.776	4.418	40	88.877	5.727	40	71.659	3.397
41	104.47	10.25	41	122.204	4.726	41	97.572	6.272	41	77.342	3.397
42	132.819	8.988	42	125.451	4.418	42	80.733	5.5	42	81.831	3.397
43	92.149	8.796	43	95.004	4.418	43	57.078	5.12	43	59.025	3.397
44	102.145	10.002	44	98.949	4.726	44	61.903	5.397	44	66.036	3.397
45	135.983	9.348	45	124.33	4.418	45	86.446	6.121	45	73.835	3.397
46	121.032	8.65	46	131.342	4.418	46	91.196	5.849	46	86.631	3.397
47	153.535	8.808	47	140.725	4.418	47	106.827	5.502	47	86.237	3.397
49	135.572	9.554	49	141.993	4.726	49	79.82	5.727	49	84.894	3.397
56	122.122	8.663	56	121.543	4.726	56	83.675	5.612	56	80.202	3.397
76	147.168	8.824	76	148.745	4.726	76	81.388	5.61	76	79.798	3.397
79	111.576	9.563	79	88.913	4.418	79	70.677	6.611	79	61.805	3.397
80	125.129	9.776	80	119.099	4.418	80	90.32	5.981	80	77.474	3.397
87	89.969	12.691	87	96.972	4.418	87	63.757	7.016	87	66.429	3.397

Table B.2. Least squares estimated, backtransformed means for survival in the mixed and separate treatments, at the subtidal and intertidal sites, sorted by family. P-values for all estimates are <0.0001.

Subtidal survival						Intertidal survival					
Mixed			Separate			Mixed			Separate		
family	survival	SE	family	survival	SE	family	survival	SE	family	survival	SE
1	60.108	8.131	1	59.925	5.05	1	85.355	10.46	1	75.722	6.873
2	44.975	8.131	2	39.263	4.721	2	37.059	10.46	2	65.456	6.873
3	50	8.131	3	60.323	4.721	3	65.748	10.46	3	76.71	6.873
4	34.448	8.131	4	57.431	4.721	4	78.426	10.46	4	82.907	6.873
5	75.96	8.131	5	70.338	5.456	5	88.534	10.46	5	92.91	6.873
6	71.534	8.131	6	59.503	4.721	6	89.668	10.46	6	84.995	6.873
8	77.157	8.131	8	75.399	4.721	8	89.528	10.46	8	87.821	6.873
9	62.929	8.131	9	64.345	4.721	9	62.721	10.46	9	78.537	6.873
10	33.576	8.131	10	52.972	4.721	10	53.156	10.46	10	81.586	6.873
11	67.566	8.131	11	65.601	4.721	11	69.751	10.46	11	80.715	6.873
12	63.035	8.131	12	62.468	4.721	12	77.684	10.46	12	77.305	6.873
13	14.645	8.131	13	47.999	4.721	13	24.423	10.46	13	89.207	6.873
14	50	8.131	14	50.488	4.721	14	78.426	10.46	14	54.771	6.873
15	66.424	8.131	15	73.756	4.721	15	69.751	10.46	15	83.989	6.873
16	55.135	8.131	16	56.383	4.721	16	78.426	10.46	16	74.541	6.873
17	39.785	8.131	17	48.249	4.721	17	75.577	10.46	17	62.511	6.873
18	65.448	8.131	18	58.083	5.456	18	85.825	10.46	18	73.908	6.873
19	67.821	8.131	19	64.551	4.721	19	65.748	10.46	19	68.791	6.873
20	62.929	8.131	20	66.161	4.721	20	66.812	10.46	20	83.233	6.873
21	71.088	8.131	21	69.39	4.721	21	88.534	10.46	21	83.345	6.873
22	52.516	8.131	22	63.883	4.721	22	75.577	10.46	22	81.08	6.873
23	55.135	8.131	23	60.363	4.721	23	69.541	10.46	23	73.941	6.873
24	57.631	8.131	24	47.879	5.05	24	53.156	10.46	24	71.829	6.873
25	73.777	8.131	25	69.929	4.721	25	83.383	10.46	25	84.604	6.873
26	39.892	8.131	26	48.659	4.721	26	56.526	10.46	26	77.018	6.873
28	50	8.131	28	57.336	5.05	28	83.383	10.46	28	56.646	6.873
29	44.975	8.131	29	55.145	4.721	29	63.586	10.46	29	86.751	6.873
32	62.665	8.131	32	72.648	4.721	32	78.426	10.46	32	78.46	6.873
33	70.599	8.131	33	62.957	4.721	33	62.721	10.46	33	86.775	6.873
34	75.96	8.131	34	71.891	4.721	34	80.966	10.46	34	89.845	6.873
35	55.516	8.131	35	61.123	4.721	35	65.748	10.46	35	69.691	6.873
36	72.866	8.131	36	51.628	4.721	36	82.181	10.46	36	73.184	6.873
37	47.484	8.131	37	49.781	4.721	37	80.438	10.46	37	81.06	6.873
39	71.534	8.131	39	67.135	5.05	39	56.526	10.46	39	87.101	6.873
40	57.631	8.131	40	61.236	4.721	40	80.257	10.46	40	94.314	6.873
41	49.89	8.131	41	51.027	5.05	41	62.721	10.46	41	69.509	6.873
42	66.32	8.131	42	59.172	4.721	42	81.666	10.46	42	81.666	6.873
43	67.566	8.131	43	62.8	4.721	43	84.697	10.46	43	89.963	6.873
44	52.133	8.131	44	62.648	5.05	44	84.697	10.46	44	79.987	6.873
45	58.387	8.131	45	53.009	4.721	45	65.964	10.46	45	83.758	6.873
46	70.25	8.131	46	54.146	4.721	46	72.611	10.46	46	75.846	6.873
47	68.534	8.131	47	59.252	4.721	47	75.577	10.46	47	85.257	6.873
49	52.516	8.131	49	44.762	5.05	49	80.257	10.46	49	49.283	6.873
56	65.811	8.131	56	73.563	5.05	56	83.552	10.46	56	82.364	6.873
76	67.821	8.131	76	59.056	5.05	76	78.974	10.46	76	89.403	6.873
79	55.135	8.131	79	48.983	4.721	79	56.3	10.46	79	68.241	6.873
80	55.406	8.131	80	68.587	4.721	80	69.751	10.46	80	85.505	6.873
87	32.179	8.131	87	37.498	4.721	87	49.772	10.46	87	67.245	6.873

Table B.3. Least squares estimated means for individual weight in the mixed and separate treatments, at the subtidal and intertidal sites, sorted by value from high to low. P-values for all estimates are <0.0001.

Subtidal individual weight						Intertidal individual weight					
Mixed			Separate			Mixed			Separate		
family	indwtg	SE	family	indwtg	SE	family	indwtg	SE	family	indwtg	SE
13	156.877	16.233	76	148.745	4.726	47	106.827	5.502	46	86.631	3.397
47	153.535	8.808	49	141.993	4.726	15	100.626	5.850	47	86.237	3.397
76	147.168	8.824	47	140.725	4.418	41	97.572	6.272	49	84.894	3.397
37	145.245	10.494	25	136.453	4.418	35	96.944	6.120	15	82.298	3.397
22	142.809	10.008	13	133.271	4.418	4	95.201	5.609	42	81.831	3.397
16	141.181	9.750	18	133.001	5.106	22	93.357	5.725	39	80.396	3.397
39	139.689	8.507	46	131.342	4.418	12	92.605	5.615	56	80.202	3.397
20	139.146	9.146	12	131.245	4.418	46	91.196	5.849	76	79.798	3.397
35	138.641	9.542	37	130.283	4.418	39	91.082	5.855	28	79.742	3.397
40	137.813	9.547	19	129.842	4.418	8	90.836	5.402	1	79.392	3.397
1	137.746	9.336	35	127.390	4.418	80	90.320	5.981	3	79.295	3.397
15	137.718	8.813	15	126.312	4.418	32	89.776	5.609	12	79.254	3.397
4	137.186	12.269	22	126.241	4.418	6	89.184	5.504	32	78.866	3.397
45	135.983	9.348	8	126.088	4.418	20	89.044	6.123	18	78.239	3.397
49	135.572	9.554	16	125.665	4.418	40	88.877	5.727	8	77.960	3.397
42	132.819	8.988	4	125.633	4.418	3	88.444	5.979	20	77.935	3.397
19	132.435	8.661	42	125.451	4.418	37	86.590	5.728	80	77.474	3.397
8	130.540	8.364	45	124.330	4.418	45	86.446	6.121	41	77.342	3.397
26	129.325	11.454	10	123.361	4.418	19	86.116	5.979	10	76.823	3.397
25	127.273	8.348	41	122.204	4.726	16	85.749	5.609	16	76.770	3.397
24	125.493	9.367	56	121.543	4.726	24	84.319	6.808	26	76.691	3.397
80	125.129	9.776	1	121.382	4.726	56	83.675	5.612	37	76.623	3.397
12	124.860	9.169	6	120.957	4.418	76	81.388	5.610	14	76.510	3.397
2	123.493	10.843	3	120.667	4.418	42	80.733	5.500	11	76.355	3.397
28	123.010	10.246	21	120.615	4.418	25	80.606	5.504	35	76.301	3.397
18	122.378	8.958	80	119.099	4.418	49	79.820	5.727	24	76.148	3.397
56	122.122	8.663	40	118.776	4.418	1	78.957	5.501	36	76.120	3.397
3	121.890	10.231	26	118.457	4.418	23	78.882	5.980	13	76.074	3.397
46	121.032	8.650	20	118.228	4.418	10	78.826	6.443	4	75.890	3.397
29	120.592	10.788	39	117.842	4.726	2	78.702	8.103	22	75.751	3.397
6	117.704	8.499	17	117.444	4.418	18	77.436	5.400	25	75.658	3.397
23	115.476	9.560	32	114.635	4.418	29	76.323	5.980	19	74.389	3.397
11	114.754	8.821	28	114.254	4.726	13	74.834	9.919	45	73.835	3.397
32	113.081	8.974	11	113.415	4.418	14	73.841	5.609	40	71.659	3.397
21	112.279	8.664	5	112.591	5.106	36	72.863	5.501	6	71.038	3.397
79	111.576	9.563	14	111.155	4.418	79	70.677	6.611	23	70.901	3.397
10	110.495	11.852	2	110.693	4.418	17	68.079	5.501	2	70.779	3.397
36	109.746	8.496	24	110.148	4.726	11	67.432	5.981	17	68.603	3.397
5	107.570	8.227	36	107.749	4.418	21	67.388	5.300	29	68.109	3.397
41	104.470	10.250	23	106.652	4.418	28	67.023	5.504	5	66.612	3.397
44	102.145	10.002	29	104.246	4.418	87	63.757	7.016	87	66.429	3.397
14	100.593	10.223	9	102.825	4.418	44	61.903	5.397	44	66.036	3.397
17	98.085	11.455	44	98.949	4.726	5	60.602	5.399	21	65.240	3.397
43	92.149	8.796	34	97.325	4.418	26	60.193	6.612	79	61.805	3.397
34	91.584	8.514	87	96.972	4.418	34	57.638	5.729	43	59.025	3.397
33	90.597	8.660	43	95.004	4.418	9	57.333	6.272	33	58.633	3.397
87	89.969	12.691	79	88.913	4.418	43	57.078	5.120	9	58.622	3.397
9	89.354	9.190	33	80.087	4.418	33	48.531	6.272	34	56.940	3.397

Table B.4. Least squares estimated, backtransformed means for survival in the mixed and separate treatments, at the subtidal and intertidal sites, sorted by value from high to low. P-values for all estimates <0.0001.

Subtidal survival						Intertidal survival					
Mixed			Separate			Mixed			Separate		
family	survival	SE	family	survival	SE	family	survival	SE	family	survival	SE
8	77.157	8.131	8	75.399	4.721	6	89.668	10.460	40	94.314	6.873
5	75.960	8.131	15	73.756	4.721	8	89.528	10.460	5	92.910	6.873
34	75.960	8.131	56	73.563	5.050	5	88.534	10.460	43	89.963	6.873
25	73.777	8.131	32	72.648	4.721	21	88.534	10.460	34	89.845	6.873
36	72.866	8.131	34	71.891	4.721	18	85.825	10.460	76	89.403	6.873
6	71.534	8.131	5	70.338	5.456	1	85.355	10.460	13	89.207	6.873
39	71.534	8.131	25	69.929	4.721	43	84.697	10.460	8	87.821	6.873
21	71.088	8.131	21	69.390	4.721	44	84.697	10.460	39	87.101	6.873
33	70.599	8.131	80	68.587	4.721	56	83.552	10.460	33	86.775	6.873
46	70.250	8.131	39	67.135	5.050	25	83.383	10.460	29	86.751	6.873
47	68.534	8.131	20	66.161	4.721	28	83.383	10.460	80	85.505	6.873
19	67.821	8.131	11	65.601	4.721	36	82.181	10.460	47	85.257	6.873
76	67.821	8.131	19	64.551	4.721	42	81.666	10.460	6	84.995	6.873
11	67.566	8.131	9	64.345	4.721	34	80.966	10.460	25	84.604	6.873
43	67.566	8.131	22	63.883	4.721	37	80.438	10.460	15	83.989	6.873
15	66.424	8.131	33	62.957	4.721	40	80.257	10.460	45	83.758	6.873
42	66.320	8.131	43	62.800	4.721	49	80.257	10.460	21	83.345	6.873
56	65.811	8.131	44	62.648	5.050	76	78.974	10.460	20	83.233	6.873
18	65.448	8.131	12	62.468	4.721	4	78.426	10.460	4	82.907	6.873
12	63.035	8.131	40	61.236	4.721	14	78.426	10.460	56	82.364	6.873
9	62.929	8.131	35	61.123	4.721	16	78.426	10.460	42	81.666	6.873
20	62.929	8.131	23	60.363	4.721	32	78.426	10.460	10	81.586	6.873
32	62.665	8.131	3	60.323	4.721	12	77.684	10.460	22	81.080	6.873
1	60.108	8.131	1	59.925	5.050	17	75.577	10.460	37	81.060	6.873
45	58.387	8.131	6	59.503	4.721	22	75.577	10.460	11	80.715	6.873
24	57.631	8.131	47	59.252	4.721	47	75.577	10.460	44	79.987	6.873
40	57.631	8.131	42	59.172	4.721	46	72.611	10.460	9	78.537	6.873
35	55.516	8.131	76	59.056	5.050	11	69.751	10.460	32	78.460	6.873
80	55.406	8.131	18	58.083	5.456	15	69.751	10.460	12	77.305	6.873
16	55.135	8.131	4	57.431	4.721	80	69.751	10.460	26	77.018	6.873
23	55.135	8.131	28	57.336	5.050	23	69.541	10.460	3	76.710	6.873
79	55.135	8.131	16	56.383	4.721	20	66.812	10.460	46	75.846	6.873
22	52.516	8.131	29	55.145	4.721	45	65.964	10.460	1	75.722	6.873
49	52.516	8.131	46	54.146	4.721	3	65.748	10.460	16	74.541	6.873
44	52.133	8.131	45	53.009	4.721	19	65.748	10.460	23	73.941	6.873
3	50.000	8.131	10	52.972	4.721	35	65.748	10.460	18	73.908	6.873
14	50.000	8.131	36	51.628	4.721	29	63.586	10.460	36	73.184	6.873
28	50.000	8.131	41	51.027	5.050	9	62.721	10.460	24	71.829	6.873
41	49.890	8.131	14	50.488	4.721	33	62.721	10.460	35	69.691	6.873
37	47.484	8.131	37	49.781	4.721	41	62.721	10.460	41	69.509	6.873
2	44.975	8.131	79	48.983	4.721	26	56.526	10.460	19	68.791	6.873
29	44.975	8.131	26	48.659	4.721	39	56.526	10.460	79	68.241	6.873
26	39.892	8.131	17	48.249	4.721	79	56.300	10.460	87	67.245	6.873
17	39.785	8.131	13	47.999	4.721	10	53.156	10.460	2	65.456	6.873
4	34.448	8.131	24	47.879	5.050	24	53.156	10.460	17	62.511	6.873
10	33.576	8.131	49	44.762	5.050	87	49.772	10.460	28	56.646	6.873
87	32.179	8.131	2	39.263	4.721	2	37.059	10.460	14	54.771	6.873
13	14.645	8.131	87	37.498	4.721	13	24.423	10.460	49	49.283	6.873