PROJECT TERMINATION REPORT
PART I: SUMMARY

PROJECT TITLE: Crossbreeding Pacific Oyster for High Yield

REPORTING PERIOD: 4/01/01 - 3/31/05

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REASON for TERMINATION: End of funding; completion of project objectives.

PROJECT OBJECTIVES: The project had distinct research and outreach components. The research component of the project had five integrated objectives for improving the efficiency of testing hybrid yield and the crossbreeding potential of inbred lines: (1) to make and maintain new inbred lines from pedigreed families produced by the Molluscan Broodstock Program (MBP), using brother-sister matings and microsatellite DNA markers to ensure pedigree (OSU, TRI, USC); (2) to test the performance of hybrids produced by factorial crosses of these inbred lines, using the MBP protocol for assessing family yields with replicated bag culture (TRI); (3) to develop and test methods for cryopreserving oyster embryos and early larvae to facilitate regional research (OSU); (4) to correlate metabolic performance or patterns of gene expression of hybrid larvae with growth to market size, to verify the major hypothesis that elite inbred lines might be identified without need for extensive field-testing (USC, TRI); (5) to map lethal genes and heterosis QTL in inbred lines and to use markers associated with them to select optimal broodstock from within and among inbred lines (USC, TRI).

The outreach component of the project has the following objectives: (1) to organize industry workshops on broodstock management and crossbreeding; (2) to work with industry to develop and use standard protocols for evaluating yield of test and control groups; (3) to maintain an inventory of available inbred lines and control broodstock populations and deliver appropriate broodstock to participating commercial hatcheries for yield trials; (4) to work with industry to develop methods for tracking broodstock and offspring through the hatchery, nursery and grow-out phases of commercial culture to harvest; (5) to create material on broodstock management, basic genetics, crossbreeding, and the WRAC project for web pages (*e.g.* MBP, UCD, USC, WRAC, PCSGA sites).

PRINCIPAL ACCOMPLISHMENTS: The achievements of this WRAC project are summarized by objective.

Research Objective 1: Making New Inbred Lines. Owing to the two-years needed to obtain fecund females reliably, production of inbred lines from crosses of brothers and sisters was done in only the first two years of the four-year project. In that time, 135 pair crosses of brothers and sisters were attempted. Approximately 95 of these crosses produced seed, which was sent to a broodstock repository on Taylor Shellfish Farm ground in Totten Inlet, South Puget Sound. Each inbred line was stocked into three cages to obtain relative yield data, and the poorer performing half of each group of lines produced was eliminated. The inventory today stands at 48 different inbred lines, 47 of which are in two or more cages, having more than >20 individuals each. Twenty-one lines, including four lines from self-fertilizations, represent the first inbred generation. Twenty-five lines are in the second generation of inbreeding (nine of these are also represented by families with one generation of inbreeding), and two lines are in the third generations of inbreeding (also represented by families with two generations of inbreeding).

In the course of this project, we typed microsatellite DNA markers to confirm parentage and pedigree for 1205 prospective parents of 26 experimental crosses, conducted in three different hatcheries, Whiskey Creek Hatchery, Taylor Resources, Inc., and the experimental culture facility at USC's Wrigley Marine Science Center on Catalina Island. By the second year of the project, we developed a set of 11 markers distributed across the 10 linkage groups of the Pacific oyster (Hubert & Hedgecock, 2004; N.B. The families used for linkage mapping were generated from stocks produced in parent WRAC projects). Of the 978 individuals, for which we kept genotyping records, 79 (8.1%) proved to have genotypes incompatible with their supposed pedigree and were rejected as contaminants. Contamination was not equally distributed across families; for 18 inbred families typed in 2003 (169 individuals), for example, we found no contaminants in 5 lines; one in 5 lines; two in 3 lines; three, four, and five in 1 line each; and six in two lines. The sources of contamination were not determined. Genotyping prospective broodstock was thus confirmed as an essential component of a commercial crossbreeding program.

Research Objective 2: Testing Hybrids in Replicated Field Trials.

At the TRI hatchery, we attempted a total of 11 factorial crosses among inbred lines to produce F_1 hybrids for yield testing. Five crosses (01x1, 01x4, 03x1, 03x4, and 03x6, where crosses are labeled by year and experiment number within the year) produced sufficiently replicated data at the seed stage for analyses of the genetic components of yield. Four crosses (01x1, 01x4, 03x6, and 03x8) produced data on adult harvest. Other crosses had poor survival in the hatchery (04x2), especially in the nursery phase when ciliates were a problem (02x2, 02x3, 04x3), or were lost to winter storms (02x1, 02x2).

Data for seed and adult yield were analyzed by ANOVA to estimate the **general combining abilities** of parent inbred lines (GCA, based on additive genetic effects), the **special combining abilities** of a particular cross (SCA, based on non-additive interaction between parent inbred lines), and differences between reciprocal hybrids (R, based on extranuclear effects causing deviations from what should be equivalent nuclear gene effects). In general, we found evidence that all three sources of genetic variation contribute significantly to oyster yield; nevertheless, the highest yields, in three of four experiments taken to adult harvest, were attributable to hybrids with high SCA and low GCA. These results suggest that improving the yield of commercial oyster seed can be achieved, in part, by selection among inbred lines, based on general combining ability, but to a greater extent by selection of crosses with the highest specific combining ability. Also noteworthy in these experiments were large differences between reciprocal hybrids, which suggest constraints on the direction in which inbred parent lines can be crossed.

To investigate the possibility that yield at harvest and its genetics components (GCA, SCA, and R) could be predicted by performance at the seed stage (i.e. at the end of the outdoor seed rearing culture phase), we measured the correlation of seed growth and final yield in three experiments (01x1, 01x4, and 03x6). In general, we obtained only mildly positive correlations in the ranks of family yields at the seed and harvest stages. Still, the correlation of SCA, the most important genetic component for crossbreeding, between seed and adult stages was highly significant in 03x6 and significant in 01x4, if an outlier was removed. The correlation between adult *vs.* seed SCA for yield in experiment 01x1 showed a negative though non-significant correlation, which was also attributable to two outliers with high seed SCA and low adult SCA. Overall, our experimental results suggested that specific combining ability for yield at harvest could be predicted by data on seed yield. This means that a commercial crossbreeding program could make progress by choosing top crosses at the seed stage, after the first growing season (if planted early enough), rather than at harvest size, after a second or third growing season.

Protocols for commercial yield-testing developed during the course of this project include: nomenclature for inbreed lines and hybrids, which facilitates tracking; sexing and biopsy of broodstock tissues; "dry storage" of broodstock oysters while awaiting genotyping results; genotyping to confirm pedigree; induction of metamorphosis with epinephrine, producing cultchless single-oyster seed; a four-stage rearing cycle (Phase 1, larval rearing; Phase II, seed rearing on indoor upwelling screens; Phase III, seed rearing in off-bottom tidally rotating pouches; Phase IV, final growout in either a suspended or on-bottom a cage); packed volume counts of seed; use of flat-sided on-bottom growout cages attached to an anchor line, permitting periodic flipping of the bag and smothering of fouling barnacles; early planting so that the oysters reach Phase IV at the end of their first summer and over-winter in large cages, which are less easily lost to storms or heavily fouled by barnacles in early spring; inclusion of a *51×35* hybrid cross as a standard cross in all yield trials.

Research Objective 3: Cryopreserving oyster embryos and early larvae. Cryopreservation of gametes from elite inbred lines would help industry to maintain the lines and facilitate their use by commercial hatcheries. The project developed a simple protocol for cryopreservation of Pacific oyster sperm, which requires no special equipment beyond standard laboratory items and can be replicated in any research or commercial setting. The protocol specifies the optimal rate of cooling (exposing 2.5 ml plastic straws to -70°C) and thawing (at 40° C), sperm concentration (a sperm-to-diluent ratio of 1:10), and prevention of sperm agglutination (with 1% polyvinyl alcohol PVA). Mean fertilization using thawed sperm from this protocol consistently ranged from 50% to 100% of the control. Sperm cooled using our protocol is concentrated and can be held at -196° C long-term.

Research Objective 4: Correlating metabolic parameters or patterns of gene expression in larvae with growth to market size. Data on larval growth data were obtained from seven experiments conducted at USC, where replication of larval cultures was possible. These seven experiments comprised 13 crosses between pairs of inbred lines, allowing for measurement of heterosis or hybrid vigor (defined as hybrid growth exceeding the growth of the faster growing inbred parent line) for 26 hybrid families. We found significant hybrid vigor for 18 of the 26 hybrids; in one other case, the hybrid was smaller than the smaller parent, a rare case of negative heterosis. Hybrid vigor was consistently expressed across different crosses of the same inbred families. Of the seven cases, in which hybrid vigor was not significant, we found four cases of dominance (*i.e.* the hybrid grew at the same rate as the faster growing inbred parent line). Thus, larval growth in these crosses showed non-additive inheritance (hybrids differed from the mid-parent value) in 88% of cases. Mechanisms accounting for greater growth of hybrid *vs.* inbred oyster larvae reared under identical environmental conditions were the focus of intense study.

Experiments at USC furnished a tremendous amount of physiological and biochemical data on the metabolic causes of growth heterosis, as well as RNA samples for evaluating patterns of gene expression underlying growth heterosis. At the physiological level, key components of the energy balance equation for larvae (Growth = Consumption – Respiration – Excretion) were investigated by a suite of integrated measurements, including hybrid *vs.* inbred larval growth, biochemical compositions, rates of nutrient acquisition (both particulate and dissolved), absorption efficiencies, metabolic rates, and enzyme activities. About one-third of the energy difference between inbred and hybrids growth rates comes from enhanced, size-specific feeding ability of hybrids. The other two-thirds of the energy difference between inbred and hybrid growth rates must be explained by differences in metabolic efficiencies, especially differences in the efficiencies of protein deposition. Perhaps, more importantly, experimental results eliminated or made unlikely some potential explanations for growth heterosis, such as differences in absorption rates, size-specific metabolic rates, or total aerobic capacity, allowing future research to be focused on key endogenous processes.

Clones of candidate heterosis genes, which were identified through comparative gene-expression profiling of inbred and hybrid oysters by massively parallel signature sequencing (Hedgecock et al. 2001, 2002, and in preparation), were transferred to USC, where 165 were successfully amplified and sequenced. Partial sequences for candidate genes enabled identification of some homologous genes in GenBank; interestingly, several proved to be ribosomal proteins, which are important in growth of yeast and Drosophila cells. Monitoring the expression levels of these candidate genes may ultimately provide an accurate early predictor of hybrid performance.

Research Objective 5: Purging lethal genes from inbred lines. We did not succeed in accomplishing this task. During the first two years, we had no second-generation inbred lines to sample. During the last two years, we were in no danger of losing important inbred lines and put limited resources into other objectives.

Outreach Objective 1: Organizing industry workshops. Work Group members gave presentations on WRAC research at the annual joint meetings of the Pacific Coast Shellfish Growers Association and the Pacific Coast Section of the National Shellfisheries Association and at the National Shellfisheries Association meetings. In addition, Sean Matson presented information about the WRAC project at the Alaska shellfish growers meeting in November 2001 and organized a joint workshop for the MBP and the WRAC projects at the Pacific Coast Shellfish Growers Association meeting in October 2002.

Outreach Objective 2: Developing standard protocols for evaluating commercial yield of hybrids. Two commercial yield trials were attempted with hybrids. The first trial, initiated in May 2001, pitted a control batch of Netarts Bay, OR, wild oysters against *51×35* hybrids, produced at the Taylor Whiskey Creek hatchery (TWC). Larvae were sent

to the TRI hatchery for cultchless setting, and then to the Taylor Shellfish Farms nursery facility in Kona, HI, for rapid production of larger seed. About 1 million seed each of hybrid and control groups were returned to the Taylor FLUPSY (floating upwelling nursery system) in Oakland Bay. The seed were planted, at 180 oysters per bag, in Spring 2002, in North Bay, South Puget Sound, for final grow-out; they were harvested in January 2004. Results suggested that *51×35* hybrids were better performing than current industry stock, despite their being inferior to several other hybrid combinations in experimental testcrosses 01x1 and 03x6. Oysters were machine graded into size categories (sub-market, extra-small, small, #4, and medium) before weighing. Although hybrid oysters had greater average weight than control oysters over all size categories and in the extra small, small, and medium categories, differences were not statistically significant. After grading of live oysters, 20 to 25 oysters from each of the hybrid and control groups, within each size category, were shucked to obtain meat wet weights, dry meat weights, dry shell weights, and a condition index (dry meat weight divided by shell weight × 1000). Again, ANOVA revealed no significant difference between hybrids and controls for any of the post-shucking harvest traits, although in all cases the hybrid exceeded the control. Post-hoc tests did reveal statistically significant differences between hybrids and controls for some size-categories. For example, wet meat weight in the extra small category was 1.6 g greater in hybrids than in controls, suggesting that hybrids might yield a plumper half-shell product.

A second, commercial-scale trial was initiated in July 2002, again using the *51×35* cross. A control batch of larvae from wild Willapa Bay broodstock was produced at TWC at the same time. The hybrid larvae set on day 14, 1 to 2 days faster than normal. Fourteen million hybrid eyed-larvae, together with 1.0-1.5 million control eyed-larvae, were sent to the Taylor Shellfish Farms site in Samish Bay, WA, for remote setting and growout on about 200, 100 ft. longlines. Unfortunately, these oysters were planted high in the intertidal and suffered a severe barnacle set in winter of 2004, which impeded their growth. They have still not been harvested, and their ultimate fate is uncertain.

Outreach Objective 3: Maintaining inventories of inbred lines available to commercial hatcheries for yield trials. The MBP website (http://www.hmsc.orst.edu/projects/mbp/index.html) listed those inbred broodstocks that were available, from cohorts 5 and 7. Other inbreds were in too limited a supply to be offered widely to industry.

Outreach Objective 4: Developing methods for tracking stock through commercial culture systems. Several meetings between TSF broodstock managers and Work Group participants focused on the development of a relational database (Microsoft Access) for maintaining inventory and tracking groups through the commercial culture system. The commercial hybrid cross and the inventory of WRAC inbred lines were tracked in this database. Both Taylor Shellfish Farms and Coast Seafoods established systems for tracking groups from hatchery to market. These systems were tested during the course of our commercial-scale test of hybrid and control seed.

Outreach Objective 5: Creating outreach material in electronic and print formats. The WRAC Crossbreeding website is http://www.hmsc.orst.edu/projects/wrac. It includes a home page with links to a project summary page, progress reports, a "primer" on genetics for the layman, and a contact page.

IMPACTS: This project gave shape and practical substance to commercial crossbreeding of Pacific oysters, fundamentally changing the industry's approach to breeding. We identified the need for a two-tiered system of testcrosses to identify elite inbred lines for commercial hybrid seed production. With maize, elite lines are identified annually by crossing thousands of inbred lines, producing millions of hybrid combinations, which are then simultaneously planted and evaluated for yield. In this way, the commercial corn breeder exerts tremendous selection for specific combining ability, taking the best pair of inbred lines out of the thousands tested. With oysters, however, we found that rearing even a 7×7 factorial crosses of inbred lines to harvest size was difficult and impractically labor intensive; indeed, we succeeded only four times (in experiments 01x1, 01x4, 03x6, and 03x8). We realized these limitations, heading into the project, which is why we proposed to evaluate testing of inbred lines at the larval or young seed stage. For the larval stage, we continued work on physiological or functional genetic indices of fast growth, as discussed above. For the young seed stage, we explored correlation of seed with final yield and of general and specific combining abilities across life stages. Because these correlations appear to be positive but weak, we recommend retention of the top inbred parent lines determined by factorial experiments, followed by re-testing of their combining abilities on a much larger production scale. This strategy is currently being followed by Taylor Resources.

RECOMMENDED FOLLOW-UP ACTIVITIES: During the course of the project, the industry experienced a marked shift in demand for triploid hatchery seed. Today, industry-wide, 40-50% of hatchery seed are triploid, and for Taylor Shellfish Farms the percentage of triploid seed is even higher, 100% for singles, 50% for shucked meat product. Thus, commercial crossbreeding program will need to focus on production of triploid rather than diploid hybrid seed. To take full advantage of non-additive components of genetic variation for yield, industry will need to incorporate specific combining ability into the tetraploid lines. This can be done by chemically inducing triploidy in the fertilized eggs of diploid hybrids and using the resulting triploids to found tetraploid stocks. The suggestion from the maize literature is that heterosis compounds with higher ploidy levels: just as AB is better than AA at the diploid level, ABC > AAB at the triploid level, and ABCD > AABC at the tetraploid level. Taylor Resources initiated retention of high-performing hybrid crosses from the 2003 factorial crosses, with the intent of utilizing hybrid females for tetraploid production (via chemically induced triploidy). Currently, Taylor Resources maintains approximately 65 F_1 hybrid families.

SUPPORT:

YEAR	WRAC-USDA Funding	OTHER SUPPORT					TOTAL SUPPORT
		University	Industry	Other Federal	Other	Total	
2001-02	$100,000	(PI FTE)	$63,134[a]	$110,000[b]		$173,134	$273,134
2002-03	$107,004	(PI FTE)	$63,134[a]	$110,000[b]		$173,134	$280,138
2003-04	$110,697	(PI FTE)	$63,134[a]	$164,000[c]		$227,134	$337,831
2004-05	$110,697	(PI FTE)	$63,134[a]	$164,000[c]		$227,134	$337,831
TOTAL	$428,398	(PI FTE)	$252,536[a]	$548,000		$800,536	$1,228,934

[a] Estimated in-kind contributions by Taylor Resources, Inc.; labor and materials for culturing oysters, based on a rate of $52 per sq. ft. per annum for the Quilcene hatchery, with 1050 sq. ft. devoted to WRAC for 6 months.

[b] Portion of total award (direct plus indirect costs) from NRICGP-USDA, grant 99-35205-8260.

[c] Portion of total award (direct plus indirect costs) from NRICGP-USDA, grant #2003-35205-12830.

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Publications in print

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Manuscripts

Hedgecock, D., J-Z. Lin, S. DeCola, C. D. Haudenschild, E. Meyer, D. T. Manahan, and B. Bowen. Massively parallel signature sequencing reveals non-additive patterns of gene expression associated with growth heterosis in larvae of the Pacific oyster *Crassostrea gigas*. Submitted to Genome Research

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Moore, M. and D.T. Manahan. 2005. Protein expression and lipid metabolism in larvae of the Pacific oyster (*Crassostrea gigas*). Journal of Experimental Marine Biology and Ecology (in preparation).

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Papers presented:

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Hedgecock, D., J.- Zh. Lin, S. DeCola, C. Haudenschild, E. Meyer, D. T. Manahan, and B.Bowen. 2002. Analysis of gene expression in hybrid Pacific oysters by massively parallel signature sequencing. Plant & Animal Genome IX Conference abstract, http://www.intl-pag.org/pag/10/abstracts/PAGX_W15.html.

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Manahan, D.T., 2002. Physiological adaptations during animal development. National Academy of Sciences, invited plenary lecture for workshop on Polar and Ocean Genomics.

Manahan, D.T., 2002. Life in the cold biosphere. Stanford University. Invited lecturer for the Abbot Memorial Lecture Series, Stanford University' Hopkins Marine Station. Presented lecture included USDA-supported data on physiological bases of hybrid vigor in oyster larvae.

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Lang, P. and C. Langdon. 2003. Optimization of sperm cryopreservation for the Pacific oyster *Crassostrea gigas*: Evaluation of cooling rate. J. Shellfish Res. 22:339.

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