Crossbreeding and Selection for Resistance to Ocean Acidification in Pacific Oysters

WA Sea Grant Program – Final Report (2015-2020)

Introduction

Increased acidification of coastal waters in the Salish Sea has resulted in reduced viability of the shellfish industry focused on the production of Pacific oysters (*Crassostrea gigas*). As Barton et al. (2015) described, commercial oyster production in the Pacific Northwest was severely curtailed as a result of high PCO_2 concentrations in seawater associated with upwelling events on the US West coast. Indeed, high PCO_2 results in reduced pH at times in coastal embayments within the Salish Sea as well, when periodic upwelling occurs. In Dabob Bay, a stratified bay in Hood Canal, regular upwelling occurs during the spring and summer months associated with northerly winds, for example. These events bring cold seawater from depth to the surface where intake lines associated with the Taylor Shellfish Company hatchery are located. During strong northerly wind events when the waters are fully stratified, the bay may "turn over," bringing deeper and colder bottom layers to the surface. Over several hours $PC0_2$ in surface waters can increase from 400 to in excess of 1500 µatm (accompanied by a decrease in pH). As a consequence of increased concentrations of dissolved carbon dioxide, the aragonite saturation state (a measure of carbonate ion concentration) may also decrease to < 1.0 in incoming hatchery water to levels that are significantly impactful to oyster larvae (Waldbusser, et al. 2013, 2015).

These perturbations have increased in frequency over the last decade as climate change impacts the Salish Sea and can result in significant deformities and mortality of oyster larvae on a routine basis. While the problem may be partially alleviated by the addition of sodium carbonate (e.g., soda ash) to the intake lines shellfish hatcheries to change the equilibrium of carbonate to bicarbonate in seawater (and increase the amount of carbonate available to calcifiers), fundamental information on the impacts of acidification is critical to evaluate in Pacific oysters and other marine shellfish.

The goal of the WASG funded project was to develop and evaluate genetically unique Pacific oyster (*Crassostrea gigas*) brood stocks for phenotypic differences associated with exposure to differing levels of acidity at two critical life history stages early in the development of this oyster. This was accomplished by measuring physiological resilience factors by proxy, namely survivorship and early growth, in embryos and larvae exposed to induced high levels of $PC0_2$ in sea water. The work was initiated at the Taylor Shellfish hatchery in 2015. In 2016-20, the work shifted to the Puget Sound Restoration Fund hatchery and laboratory facilities located at the NOAA NW Fisheries Science Center in Manchester, WA, jointly operated at that time with Baywater, Inc. and later Pacific Hybreed, Inc.

Development of Genetic Lines

Taylor Shellfish Company supported the development of a crossbreeding program directed by J. Davis and D. Hedgecock from 2001 -2015. During that time frame, protocols were developed that established the utility of crossbreeding in Pacific oysters for increased yield (Hedgecock and Davis, 2007). The breeding program is based on a multi-generation effort to produce pedigreed oyster lines that are subsequently mated to produce commercial oyster seed. In brief, G0 generations of oysters are first produced by pair mating a single male and female oyster taken from naturalized populations in the Pacific Northwest. G0 lines are reared to maturity (12-15 months) and a brother/sister from each of the G0 lines made to produce a cohort of partially inbred lines. The inbred lines are subsequently reared to maturity (15-24 months). Utilizing males from one inbred line and females from a second, unrelated inbred line, an intraspecific hybrid line is produced for field testing (this is referred to as a test cross). The test cross typically consists of mating multiple inbred lines in all possible combinations in a full diallel mating scheme. Following 6-12 months of field testing in replicated arrays at intertidal farm sites, high performing lines are evaluated for both specific and general combining ability (Hedgecock and Davis, 2007). Unrelated lines having high combining ability are then mated (males from one line to females from another line) to produce commercial seed cohorts in a double hybrid cross. After 2015, the crossbreeding program initiated by Taylor Shellfish shifted to Pacific Hybreed, a new commercial entity focused on genetic improvement through brood stock and seed production in clams and oysters.

The opportunity afforded by WSG funding in 2015 enabled PI's to dovetail the production of a routine test cross at the Taylor Shellfish hatchery with acidification exposure trials at two critical life history stages in Pacific oysters. Because Taylor Shellfish Company was supporting research on crossbreeding in oysters at that time, there was the opportunity to utilize expertise at the University of Washington to evaluate different genetic lines for their resilience to acidification during two discrete parts of the larval life cycle, namely early embryogenesis to the veliger stage and during the transition from the larval to the juvenile stage (settlement and metamorphosis).

Production of Intraspecific Hybrid Lines

All previously produced and pedigreed inbred lines produced in the crossbreeding program had been reared at the Baywater Shellfish Farms farm site at Thorndyke Bay (Hood Canal) before being introduced to the conditioning system at the hatchery. In July 2015, individual oysters (N=30) were placed into a reproductive conditioning system at the hatchery. The system consisted of tanks of flowing seawater maintained at a temperature of 22 °C. Oysters were fed a mix of cultured microalgae produced by the hatchery. After approximately 2 weeks exposure to the warm seawater and plentiful feed oysters had fully developed reproductive tissues and were poised to spawn. Prior to executing the test cross all oysters selected for the cross were genotyped to confirm their pedigree. Details of genotyping protocols can be found in Hedgecock and Davis (2007). In August 2015 seven inbred oyster lines that had been conditioning at the Taylor Shellfish were selected from a larger group of inbred lines. Individual oysters from each inbred line

were shucked and the sex identified. An advantage associated with breeding in Pacific oysters is the capacity to strip viable gametes from individuals and use the gametes later in controlled matings. Once males and females were identified and confirmed to their pedigree, controlled crosses were made between an individual male and female from each line mated to all other individuals from the other six lines utilizing a full factorial mating scheme (Table 1). The mating scheme therefore produced 42 hybrid lines (including reciprocals) plus 7 double inbred lines for a total of 49 lines.

Fertilized embryos for all the lines were subsequently placed in a larval rearing system (7 L flow through tanks) in duplicate at the Taylor hatchery and reared following industry protocols for the production of Pacific oysters. The cohort of 42 hybrid and 7 second generation (G2) inbred lines represent a test cross and is referred to as 15x4 in the subsequent discussion (Table 1). Line designations refer to the year and family label for each line. For example, 13x5.019 refers to the 19th pair mated G1 inbred line produced in 2013.

	13x5.024x.042	13x5.019	12x3.062	12x3.028	08x3.027	08x2.034	08x2.015
13x5.024x.042	X	X	X	X	X	X	X
13x5.019	X	X	X	X	X	X	X
12x3.062	X	X	X	X	X	X	X
12x3.028	X	X	X	X	X	X	X
08x3.027	X	X	X	X	X	X	X
08x2.034	X	X	X	X	X	X	X
08x2.015	X	X	X	X	X	X	X

Table *1. Full diallel crosses made between individual male and female oysters from seven inbred lines. Forty-two hybrid (including male by female and female by male) were made plus 7 G_2 inbred lines were established.*

Acidification Screening of embryos from 15x4

For the production of lines used in the acidification exposure trials the following protocols were used. In all cases, extra sperm and egg tissue had been stripped and reserved for each of the individuals utilized. These tissues were immediately transported in a cooler with gel packs to the PSRF hatchery in Manchester. There*, additional gametes from the same set of oysters* utilized for the 15x4 test cross earlier that day were used at the PSRF laboratory to reformulate the same set of crosses. Fertilized eggs were maintained in 1L beakers in untreated seawater prior to exposure to the high $PC0_2$ treatment.

Experimental Apparatus

The acidification exposure apparatus designed and built for this project consisted of a fiberglass tank (volume approx. 200L) fit with titanium immersion heater to supply warm seawater on a flow through basis. One tank served as a control and a second identical tank served as the experimental unit. Each tank was fit with multiple sections of PVC pipe (12' long x 3" diameter) that served as culture units (N=42). The end of each pipe section was fit with a 20 micron Nytex screen necessary to retain fertilized embryos in solution. Pipe sections were placed vertically in the water column with each pipe section receiving a slow flow of ambient seawater (approx. 250 ml per minute) via gravity feed from a head tank positioned above the control tank containing the culture units.

To deliver seawater having an addition of dissolved carbon dioxide, a second tank containing an identical set of PVC sections was utilized. In this case, carbon dioxide contained in a tank of mixed gas was delivered via injection pump into the head tank and the desired level of dissolved carbon dioxide concentration achieved via regulation of the pH in the head tank. In this case, a Durafet pH sensor were placed into the head tank and connected to a pH controller (Honeywell Corporation). Because pH changes with the addition of dissolved CO_2, the controller activated a solenoid valve that regulated the amount of mixed gas entering the water stream supplying the head tank, according to the ambient pH. For the work with Pacific oyster embryos, the desired pH to be maintained was approximately 0.5 pH unit below ambient pH for the period of experimentation.

For the OA stress response work in embryos, the full set of 15x4 hybrid lines (42 total) was introduced to high $PC0_2$ and control (ambient $PC0_2$) seawater at the PSRF facility as described above. Each section of PVC was immersed to a depth of approximately 12 cm. in the control and experimental tanks, respectively. On a flow through basis, the volume of the culture unit was therefore approximately 700 ml. To each culture unit (PVC pipe with 20µm screen) a standard allocation of fertilized eggs was added to approximate an initial egg density of about 30 fertilized eggs per ml, or about 20,000 embryos per culture unit. After 36 hours all surviving embryos for all crosses from treatment and control replicates were immediately preserved in a solution of 10% formalin in well plates for later analysis of survivorship from fertilized egg to the D-hinge stage.

Acidification Screening of Pediveligers from 15x4

Approximately 16 days later an OA stress response was similarly measured in replicated cultures at the PSRF facility using pediveliger larvae obtained from the 15x4 cohort reared in Quilcene. In this case, late term larvae for a number of lines that had survived to the pediveliger stage were transported to Manchester and established in replicate cultures under flow through conditions, similar to that described above. In this case, experimental chambers were fit with 180 micron Nytex screen, suitable for the size of setting oysters. $PC0_2$ conditions were similar to those established for embryos earlier in the experiment. Pediveligers were established in individual replicate chambers following exposure to an epinephrine (to stimulate settlement) and enable the oysters to

metamorphose and develop to the spat stage in the replicated chambers. Unfortunately, due to the failure of an immersion heater that resulted in loss of temperature control for the experimental tank, the experiment was terminated after six days. At that time, all surviving oysters from all replicates were preserved for later analysis of survivorship, settlement rate and early spat growth.

Oyster seed from surviving lines of the Taylor Shellfish hatchery portion of 15x4 were subsequently out planted in Thorndyke Bay for growth to adulthood and use in further breeding.

Results of Acidification Screening – Embryos

Carbonate conditions for Control and Experimental Groups of embryos are described in Table 2. pH was approximately 0.55 units lower in the control compared to the experimental unit over the 36 hour experimental time frame. Temperature, salinity and total alkalinity conditions were otherwise similar for both control and experimental units.

Low PCO2 during Embryogenesis

Salinity (PSI)	Temperature °C	pH	Alkalinity
30	25.4	8.01	2092.58ppm
30	25	8.07	2125.30ppm
30.9	24.5	8.04	2061.91ppm

High PCO2 during Embryogenesis

Salinity (PSI)	Temperature °C	pH	Alkalinity
30.25	25.05	7.45	2093.81ppm
30	24.9	7.45	2082.53ppm
30.25	24.9	7.52	2061.95ppm

Table 2. Temperature, salinity, pH and alkalinity conditions associated with control and experimental tanks holding embryos form 42 lines of oysters following the creation of testcross 15x4.

Embryos of Pacific oysters that had been exposed to a high carbonate environment following fertilization were assessed for survivorship to the straight hinge or veliger stage. Sampling consisted of taking a uniform aliquot of volume of preserved larvae for each experimental cross for experimental and control and enumerating all surviving veligers in that volume of sample and therefore represent relative abundance compared to the relative abundance of veligers in other lines. On this basis, significant variation in survivorship to the veliger stage was observed for both control and experimental tanks

(Table 3). In the Control Group, the female from Line B (08x3.027) produced significantly greater numbers of veligers across all six females (mean =74.46) compared to the male from Line D (08x2.015) (mean across six females = 47.00). Conversely, females from all seven lines were generally comparable across the six males they were mated with. Also, differences in raw survivorship values were observed for reciprocal crosses. For example, Line AB (male A x female B) in the low carbonate environment had a survivorship value of 78.5 vs a value of 98.5 in its reciprocal cross male B x female A). Under high PCO_2 conditions similar variation in mean male and female performance was noted but was different than the pattern observed for the control conditions. In this case, variation between female lines was observed. For example, the female from Line G (12x3.062) was exhibited reduced survivorship across all males it was mated with (mean performance = 25.7) compared to female from Line A (12x3.028) (mean performance = 67.6). In this case, crosses with this female with all of the males in the matrix were better surviving compared to the female from Line G (12x3.062). Confounding these results are possible maternal effects associated with the eggs produced from different females. Additional analysis of the data set is necessary to tease out this possible effect.

		LOW CARBONATE (CONTROL)						
LINE	A	B	C	D	E	F	G	Sire Mean
A		78.5	64	80.25	82	64.75	65.25	**72.46**
B	98.5		67.25	81.75	63	71.75	65.5	**74.63**
C	68.25	50.75		70	48.75	76.25	63	**62.83**
D	24.25	58	69.5		29.5	60.5	40.25	**47.00**
E	55.5	63.5	57.75	56.75		66.25	66.5	**61.04**
F	76.25	66.25	62.5	62	63		59.75	**64.96**
G	82.75	61.75	79.25	58.25	66.5	61.25		**68.29**
Dam Mean	**67.583333**	**63.125**	**66.708333**	**68.166667**	**58.791667**	**66.791667**	**60.041667**	

		HIGH CARBONATE (EXPERIMENTAL)						
LINE	A	B	C	D	E	F	G	Sire Mean
A		39.75	41.75	50.75	51.75	46.25	24.25	**51.61**
B	66.5		46	56.75	55.5	39.25	21.5	**52.90**
C	48.25	27.25		31	31	68	19.75	**53.76**
D	19.75	29.25	62.25		25.25	56.75	17.75	**56.90**
E	68.5	65.5	73	54.3		70.5	36.5	**62.11**
F	73.5	72.75	50.25	67	59		34.5	**62.34**
G	95.75	83.75	88	58.5	76	64.25		**63.66**
Dam Mean	**62.041667**	**53.041667**	**60.208333**	**53.05**	**49.75**	**57.5**	**25.708333**	

Table 3. Relative survivorship in intraspecific lines of Pacific oysters from a full factorial diallel cross exposed to an increase in dissolved carbon dioxide during early embryogenesis.

Results of Acidification Screening – Pediveligers

Control and experimental conditions of pH are described in Table 4 for the conditions associated with the exposure of pediveligers to elevated PCO_2 under laboratory

conditions. After six days of growth following settlement and metamorphosis of the hybrid oyster lines used in the acidification trials, at least 100 surviving spat were counted and measured for size at age. Similar to the analysis of line response to acidification exposure, pediveligers exposed to high $PC0_2$ conditions over a six day period exhibited variable responses. In general, performance of pediveligers exposed to the $PC0_2$ challenge trended smaller after six days of exposure but this result was not statistically significant (Figure 1). Similar to results for embryogenesis, there was variation observed for different family lines measured but the differences were insignificant for the same line whether the line was exposed to increased carbonate or not (Figure 1). On the other hand, overall differences in growth at age were observed for different genetic lines. For example, HL 19 (15x4.062x.019) and HL 47 (15x4.062x.028) exhibited increased size at age after six days compared to all the other hybrid lines. HL 47 High in fact exhibited 46% greater growth on average (538 µm after six days) compared to HL 67 High (249 µm after 6 days) (Figure 1), indicating that line differences in response to overall levels of acidification at this early life history stage are present. Unfortunately, a malfunction in a titanium heater resulted in the termination of the study after only 6 days. The experiment was designed to run for 14 days as this is the time required for pediveliger oyster larvae to uniformly reach the spat stage and exhibit shell accretion typical of juvenile oysters.

Table 4. *Temperature, salinity, pH and alkalinity conditions associated with control and experimental tanks holding embryos form 42 lines of oysters following the creation of testcross 15x4.*

Low PC02 during Settlement

Salinity	Temperature	pH	Alkalinity
30.00	25.40	8.01	2092.58
30.00	25.00	8.07	2125.30
30.90	24.50	8.04	2061.91

High PC02 during Settlement

Salinity	Temperature	pH	Alkalinity
30.25	25.05	7.45	2093.81
30.00	24.90	7.45	2082.53
30.25	24.90	7.52	2061.95

Seed Production Evaluation for Additional Breeding

In January 2016 the seed from 15x4 that had been maintained at Thorndyke Bay (Hood Canal) was assessed for growth and survivorship (Figure 2). While some number of replicates among some lines were lost due to a winter storm event, adequate numbers of replicates still enabled an analysis of performance to be made based on adequate numbers of surviving oysters (Figure 2).

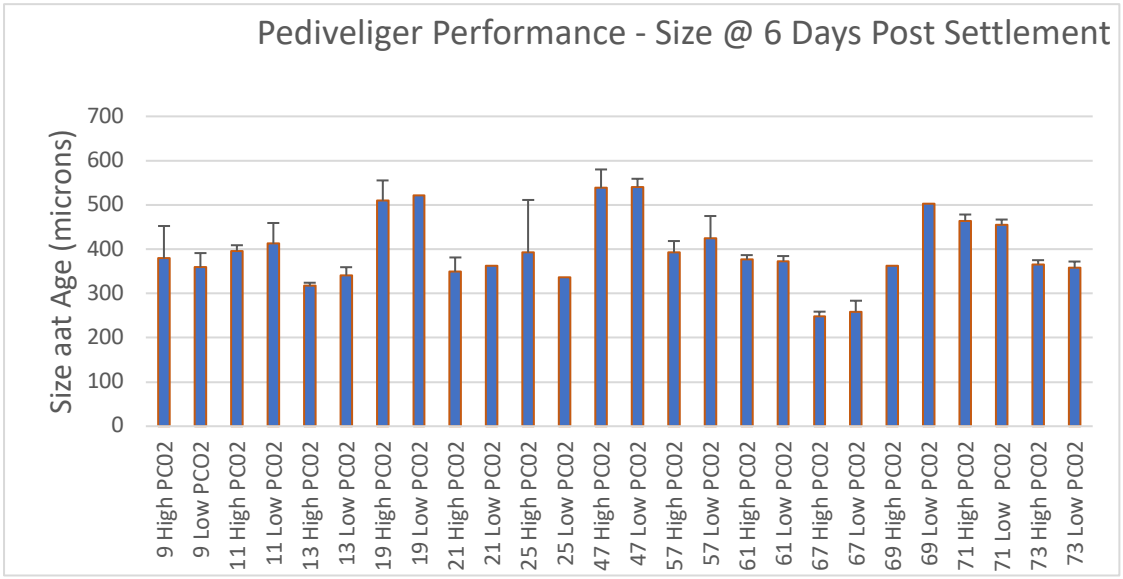

Figure 1. Results of screening pediveligers exposed to elevated PCO_2 under laboratory conditions (mean plus Std. dev.) for lines surviving to the pediveliger stage produced as part of the 15x4 testcross.

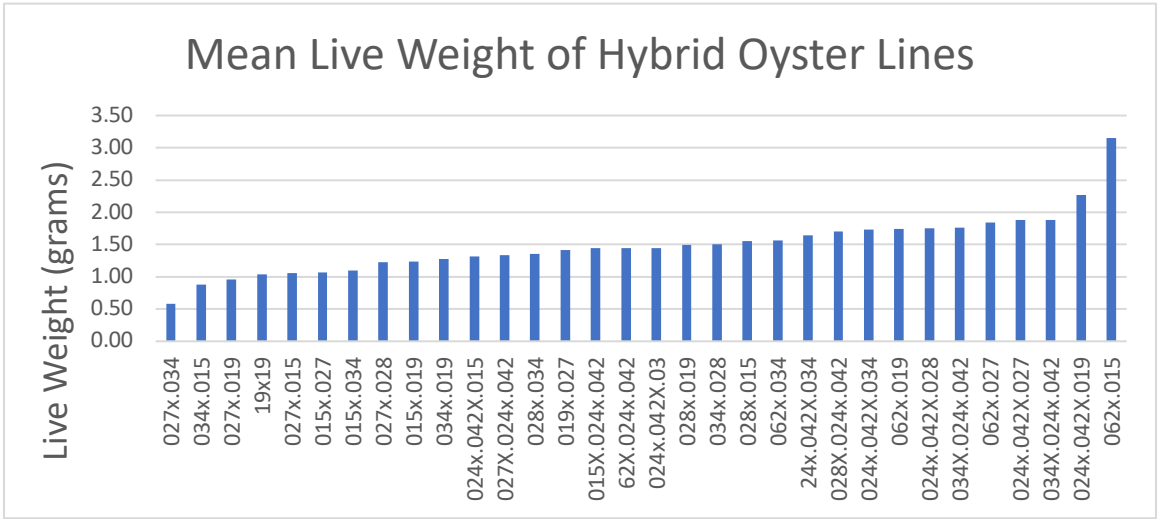

Figure 2. Results of line testing accomplished in April 2016 for surviving lines maintained at Thorndyke Bay, Hood Canal on the Baywater Shellfish Company farm site.

	13x5.024x.042	13x5.019	12x3.062	12x3.028	08x3.027	08x2.034	08x2.015
13x5.024x.042	0	5	0	4	5	5	2
13x5.019	0	5	0	1	1	0	0
12x3.062	2	5	0	0	3	2	1
12x3.028	3	5	0	0	0	4	1
08x3.027	3	4	0	3	0	3	2
08x2.034	3	2	0	1	0	0	2
08x2.015	3	1	0	0	2	2	0

Figure 3. Surviving lines (number of replicates indicated) to the seed stage assessed for general and specific combing ability. Each replicate cage of oysters contains 75 oysters for use in further breeding (pending confirmation via genotyping).

A preliminary analysis was made based on a Bayes Diallel statistical approach. Results indicated that high general combining ability was observed in lines involving parents 62 and 24 with high specific combining ability observed in a number of other crosses (D. Hedgecock, personal communication). The indication therefore is that genetically distinct lines having evidence for both General Combining Ability (GCA) and Specific Combining Ability (SCA) will be available to make high performing double hybrid crosses for the production of commercial oyster seed.

Results of a second line assessment conducted by Taylor Shellfish and PSI in April 2016 indicate that surviving lines continued to grow and otherwise thrive at Thorndyke Bay, Hood Canal (Figure 3). Due to low numbers of surviving lines, it was determined to maintain all the oyster lines at this location for final assessment and use. (spring-summer 2017).

Use of 15x4 Lines for Production and Dissemination to Industry for Further Testing

On the breeding side of the project, Pacific Hybreed attempted to spawn and rear F2 families associated with the original 15x4 cohort in 2019 and 2020. Specifically, brood stock maintained at the Thorndyke Bay repository were brought into the Pacific Hybreed hatchery laboratory in late summer in both years with the intent to spawn and rear remaining hybrid lines associated with the original 15x4 cohort. In this case, an F2 hybrid generation was the goal with the specific intent to recapture genetic material associated with the original inbred lines. In this case, the breeding design called for selecting a male and a female from each hybrid line and crossing these individuals to make F2 hybrid lines. A previous analysis, based on a Bayes Diallel statistical analysis, we intended to focus on lines demonstrating high general combining ability. These include the parent, 62 and 24

from the original 15x4 cohort, among others. There remained up to ten lines, involving these two parents, in the field. Oysters from each of these lines were brought into the hatchery for conditioning in summer 2019 and 2020. therefore, be involved in F2 line development in 2020. Unfortunately, in September 2019 there was a failure at the hatchery level involving brood stock conditioning, including a spawn out event involving the oyster brood stocks needed for F2 line development. As a result, the decision was made to conduct these crosses in spring 2020. In 2020, a hatchery closure due to the Covid 19 pandemic limited our capacity to make F2 lines. Plans for the remaining six months of the project (ending August 31, 2020) had included 1. correlating line performance relative to OA resistance based on samples taken in 2016 and 2. replicating lines for dissemination to the shellfish industry for use as brood stock. 3. spawn individuals from select hybrids (based on growth, survivorship and OA resilience at the larval and early post-larval level) to create novel F2 lines for immediate brood stock and future industry use. Unfortunately, Covid-19 restrictions prevented these activities from occurring. Instead, attempts were made to utilize the remaining 15x4 lines for commercial seed production. These attempts also failed due to problems in the rearing environment at the hatchery. Further attempts to utilize remaining 15x4 lines for F2 production and commercial double hybrid seed production will be made following the conclusion of the project.

Outreach to Industry Partners

Pacific Hybreed has been active in utilizing the 15x4 brood stock oysters associated with this project in a number of ways. First, commercial seed oyster production has been accomplished using specific hybrid crosses and disseminated to industry partners for evaluation free of charge. The utility of using oyster lines potentially resilient to acidification (e.g., use of family lines identified as better thriving in acidification trials associated with the $PC0_2$ challenge in pediveligers) is ongoing.

Public outreach of the research described here was delayed due to the unavoidable loss of expertise at the University of Washington originally committed to the project. Also, at this time, the impacts of acidification are not generally apparent in juvenile and adult oysters although some indications of increased shell dissolution have been recently noted by industry partners. At the moment, the industry is relying on buffering incoming seawater in hatcheries to reduce or eliminate the impacts of increased $PC0_2$ concentrations impacting Pacific oyster larvae. The major accomplishment of the research described here was the capacity for partnering with researchers to evaluate different genetic lines for the potential capacity to exhibit reduced stress response (evaluated as early spat growth in oysters). The work is ongoing with the necessity for additional resources to focus on evaluating overall stress response in oysters and other shellfish to increased temperature stress and susceptibility to disease.

Literature Cited

Barton, A., Waldbusser, G.G., Feely, R.A., Weisberg, R.G., Newton, J.A., Hales, B., Cudd, S., Eudeline, B., Langdon, C.J, Jeffords, I., King, T., Suhrbier, A. and McLaughlin, K. 2015. Impacts of coastal acidification on the Pacific northwest shellfish industry and adaptation strategies implemented in response. In: Special Issue on Emerging Themes in Ocean Acidification Science Oceanography, 28:pp146-159.

Hedgecock, D. and Davis, J.P. 2007. Heterosis for yield and crossbreeding of the Pacific oyster *Crassostrea gigas.* ISGA9 special issue of Aquaculture 272S1: S17–S29.

Waldbusser, G. G., Brunner, E. L. Haley, B. A. , Hales, B., Langdon, C. J., Prahl, F. G. 2013. A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. Geophysical Research Letters 40: 2171-2176.

Waldbusser, G.G., Hales, B., Langdon, C.J., Haley, B.A., Schrader, P., Brunner, E.L., Gray, M.W., Miller, C.A., and Gimenez, I. 2015. Saturation-state sensitivity of marine bivalve larvae to ocean acidification. Nature Climate Change 5: 273-280.