Examining multigenerational effects of ocean acidification on the Pacific oyster: evidence of selection or plasticity?

Daniel Jacob Gillon

A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2018

Committee:

Carolyn S. Friedman

Steven B. Roberts

Kerry A. Naish

Jonathan P. Davis

Program Authorized to Offer Degree:

Aquatic and Fishery Sciences

University of Washington

Abstract

Examining multigenerational effects of ocean acidification on the Pacific oyster: evidence of selection or plasticity?

Daniel Jacob Gillon

Chair of the Supervisory Committee:

Carolyn S. Friedman

School of Aquatic and Fishery Sciences

Marine calcifying invertebrates must be capable of acclimating or adapting to adverse carbonate conditions in order to withstand current and projected ocean acidification (OA). For some species, parental exposure to elevated CO_2 during gametogenesis may alleviate the detrimental effects of OA on larval and juvenile offspring, through transgenerational phenotypic plasticity and/or rapid selection for beneficial genotypes. Whether these patterns persist in offspring into adulthood, or across multiple generations, remains largely unknown. In a previous study by our lab group, pre-exposure of G0 adult Pacific oyster, *Crassostrea gigas*, to 1977 μatm pCO_2 prior to spawning was found to enhance the growth and survivorship of G1 larval and juvenile offspring at ambient pCO_2 levels, but did not buffer offspring from acute, negative OA effects. In this study, we assessed performance of offspring at adulthood, and reared a subsequent G2

generation at two CO_2 levels (494 and 1601 μatm). G1 adults from CO_2-exposed parents experienced reduced shell and somatic growth compared to controls, with no evidence of adaptive transgenerational effects. G2 larvae were smaller and had higher rates of mortality and shell deformities under OA conditions, though transgenerational exposure in grandparents or parents appeared to partially alleviate the effects observed at elevated pCO_2 on larvae with no history of exposure. We found evidence of maternal and paternal genetic variation in reproductive success at both high and low pCO_2 that suggests that both selection and phenotypic plasticity underlie transgenerational effects of OA on G1 and G2 oysters. More broadly, our results highlight the utility of multigenerational studies in providing a more accurate measure of populations' ability to respond to OA.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to first acknowledge my advisor, Dr. Carolyn Friedman, for her guidance, patience and encouragement over the course of a challenging graduate school journey.

I am also grateful for the support of my graduate committee: Drs. Jonathan Davis, Steven Roberts, and Kerry Naish, who assisted me in experimental design, statistical analysis and data interpretation, all while lending their sense of humor to our regular meetings.

This project would not have been possible without collaboration with the West Coast shellfish industry. I am grateful to Taylor Shellfish, Inc., particularly Benoit Eudeline, Molly Jackson, and Stuart Thomas, for allowing me to conduct research at their hatchery and for hands-on assistance in various aspects of this project. I am also grateful to Baywater, Inc., Caleb Davis and crew, for rearing and transporting oysters used in our experiments. I thank Betsy Peabody of Puget Sound Restoration Fund for devoting resources to my project.

Several collaborators were instrumental in carrying out this project: Dacey Mercer and Dr. Brett Dumbauld of USDA/ARS were instrumental in experimental design. Dacey provided invaluable assistance in genotyping and parentage analysis throughout this project.

There are numerous other individuals who assisted me in the field, in the lab, or somewhere in between: Lisa Crosson, Sam White, Bryanda Wippel, Molly Roberts, Matt George, Kristin Saksa, Windy Patten, Kyla Bivens, Ryan Fox-Horn, Robyn Strenge, Dr. Dennis Hedgecock, Stuart Ryan, Alice Helker.

Finally, I would like to thank my mom and dad for instilling in me a love for the ocean, a passion for science and an insatiable appetite for bivalves.

1. INTRODUCTION

Since the industrial era, the oceans have absorbed approximately 40% of anthropogenic CO_2 emissions (IPCC 2013). The chemical reaction caused by the uptake of CO_2 decreases the availability of carbonate ions (CO_3^{2-}) and reduces pH, a process known as ocean acidification (OA). Mean global ocean pH has declined by approximately 0.1 units over the past two centuries, corresponding to a 26% increase in acidity (IPCC 2013), and is expected to decrease by an additional 0.14 to 0.4 units by 2100 (Doney et al. 2009a). Determining the consequences of OA for marine organisms is therefore critical to predicting and managing ecosystem changes under projected climate scenarios.

Considerable research over the last decade has investigated the acute, biological effects of exposure to simulated near-future carbonate conditions on marine organisms that form calcium carbonate shells (Reviewed in Doney et al. 2009, Kroeker et al. 2010). While these effects vary across taxa, it is well documented that many marine calcifiers undergo periods of heightened vulnerability to OA during planktonic larval stages (Gazeau et al. 2013), particularly during embryogenesis and initial formation of larval shells or skeletal structure (Kurihara 2008, Byrne 2011, Barton et al. 2012, Waldbusser et al. 2013). In molluscs, observed heightened susceptibility may be the result of larvae lacking sufficient energetic reserves to meet the metabolic cost of calcification in seawater undersaturated with respect to aragonite ($\Omega_A<1$), the soluble form of calcium carbonate used during larval shell formation (Waldbusser et al. 2013, 2015a, 2015b). While acute exposure studies have provided a strong foundation for assessing the potential impacts of OA on marine organisms, our ability to predict winners and losers in

an acidifying ocean is hindered by a paucity of information on the ability of natural populations to acclimate or adapt to OA (Pespeni et al. 2013).

In the absence of new mutations, adaptation to OA may arise through selection on standing genetic variation (Kelly and Hofmann 2013). In larval sea urchin *Strongylocentrotus purpuratus,* exposure to elevated pCO_2 resulted in significant changes in allele frequency at loci responsible for biomineralization, lipid metabolism, and ion homeostasis, suggesting that selection acted on functional proteins involved in OA response (Pespeni et al. 2013). In oysters, Parker et al. (2011) found that *Saccostrea glomerata* larvae selectively-bred for fast growth outperformed wild-type conspecifics when reared under high pCO_2. Larvae of the Pacific oyster, *Crassostrea gigas*, spawned from controlled crosses of pedigreed genetic lines, varied dramatically in shell height during first-shell formation at aragonite undersaturation, with some crosses lacking a decrease in shell size under experimental conditions (Frieder et al. 2017). In juveniles of the mussel *Mytilus chilensis,* the effect of acute exposure to 979 μatm pCO_2 on calcification rate was found to differ dramatically between two populations based on the ambient chemistry conditions in natal estuaries from which broodstock were obtained prior to spawning, with one population exhibiting 0% reduction in calcification (Duarte et al. 2014). These results broadly suggest that natural populations of marine calcifiers may harbor sufficient genetic diversity to withstand OA.

Populations may also be capable of responding to OA through phenotypic plasticity within and across generations. Most marine invertebrates exhibit complex life-cycles, undergoing several dramatic ontogenic and ecological shifts over the course of fertilization, larval development, metamorphosis and maturation (Podolsky and Moran

2006). Consequently, the effects of environmental conditions experienced in one life-history stage may "carry over" into subsequent stages (Podolsky and Moran 2006, Dupont et al. 2013, Gobler and Talmage 2013, Hettinger et al. 2013). For example, Hettinger et al. (2013) found that, when reared at ~1100 μatm pCO_2 as larvae, juvenile Olympia oyster, *Ostrea lurida,* exhibited a 41% reduction in shell growth rate compared to juveniles that had been reared under control conditions as larvae. Similarly, survivorship of juvenile hard clam, *Mercenaria mercenaria*, was reduced when exposed to 1500 μatm pCO_2 during larval development (Gobler and Talmage 2013). An effective model of the impacts of OA on marine calcifiers must therefore account for latent environmental effects of one life-history stage on performance in subsequent stages.

The environmental conditions experienced by parents prior to spawning may influence the ability of offspring to withstand OA through transgenerational plasticity (TGP), in which parental environment affects the phenotypic reaction norm of offspring without altering DNA sequences (Salinas et al. 2013). TGP, which manifests as a parent environment by offspring environment interaction (Mousseau and Fox 1998), may occur due to environmentally-induced variation in maternal provisioning (Marshall and Uller 2007), or through epigenetic modifications in gene expression (i.e. DNA methylation; Razin and Riggs 1980, Gavery and Roberts 2014). The prevalence of TGP in response to OA has been investigated in marine invertebrates (Reviewed in Ross et al. 2016). For example, long-term (70d) parental pre-exposure of the sea urchin *Psammechinus miliaris* to elevated pCO_2 (999 μatm) ameliorated negative effects of OA on fertilization success, and resulted in larger offspring than were produced by parents with shorter (28d, 48d) or no pre-exposure time (Suckling et al. 2015). In the Sydney rock oyster, *Saccostrea*

glomerata, broodstock that experienced elevated pCO_2 (856 μatm) during gametogenesis produced larvae that grew more rapidly and to a larger size than larvae from control parents, which the authors attributed to increased maternal provisioning of the egg (Parker et al. 2012). Positive transgenerational effects of the F0 parental exposure were persistent in F2 larvae and juveniles (Parker et al. 2015). On the other hand, TGP is not always beneficial; pre-exposure of adult sea urchin *Strongylocentrotus droebachiensis* to elevated pCO_2 (1200 μatm) for 4 months prior to spawning resulted in a five- to nine-fold reduction in larval settlement success (Dupont et al. 2013). In sum, TGP in response to elevated pCO_2 may serve as a crucial means of shielding offspring to adverse environmental conditions over shorter time scales than are required for evolutionary adaptation, but in other instances may in fact exacerbate the negative effects of OA on offspring fitness. While disentangling these conflicting results poses a challenge to researchers, these findings nevertheless highlight the importance of transgenerational studies as a step towards a mechanistic understanding of OA impacts on marine fauna.

To date, there have been no published studies documenting transgenerational effects of OA in the Pacific oyster, *Crassostrea gigas*. This species is critical to the U.S. West Coast aquaculture industry, which generates over $100 million in annual revenue (USDA 2013). Culture of *C. gigas* in the region largely relies on larval production in land-based hatcheries, which, over the past decade, have regularly experienced catastrophic larval mortality (up to 100%) linked to upwelling of seawater with low Ω_A (often <1), resulting in region-wide shortages of seed oysters (Barton et al. 2012, 2015). Consequently, the acute effects and mechanisms of OA exposure on *C. gigas* larvae, post-larvae and adults have been heavily investigated and are well characterized, and

include reduced developmental rates, increased shell abnormalities, impaired physiological function and reduced survivorship (e.g. Kurihara et al. 2007, Lannig et al. 2010, Barton et al. 2012, 2015, Dickinson et al. 2012, Timmins-Schiffman et al. 2013, Waldbusser et al. 2013, 2015a, 2015b).

In 2013, Friedman et al. (*in prep.*) investigated the transgenerational effects of OA exposure on growth and survival in first generation (G1) larval and post-larval (~9.5 mo. post-fertilization) *C. gigas* spawned from broodstock (G0) that were reproductively conditioned at either elevated (~2000 μatm) or ambient (~1000 μatm) pCO_2. When reared at ambient pCO_2, larvae from parents conditioned at elevated pCO_2 (treatment HL) had greater survival and reached competency to settle faster than larvae from control parents (LL). After metamorphosis, HL juveniles exhibited similar growth rates and survivorship to control oysters through 30 days post-fertilization. However, by 9.5 months, HL oysters exhibited reduced shell and somatic growth relative to controls. Surprisingly, parental exposure to elevated pCO_2 did not enhance the ability of offspring to tolerate elevated pCO_2, but rather resulted in synergistically negative effects on growth and survivorship in both larvae and juveniles when larvae were reared at ~1500 μatm. These observations ran counter to expectations of an acclimatory or adaptive response, as have been observed in the Sydney rock oyster, *Saccostrea glomerata* following a similar transgenerational exposure to OA (Parker et al. 2012).

In this study, we extend the assessment of transgenerational effects of OA exposure to *C. gigas* G1 adults and second-generation (G2) larvae. The goals of this study were to determine: (1) whether transgenerational effects observed in G1 larvae and post-larvae persist into adulthood and across multiple environments, (2) whether G0

transgenerational effects are transmitted into G2 larvae, and (3) whether growth and survivorship of G2 offspring is affected by G1 larval rearing environment. In addition, we utilized molecular markers to assess variation in performance of different genetic lines following transgenerational exposure to elevated pCO_2.

Results from this study will shed light on the means by which oyster populations will respond to OA, and may inform hatchery protocols to improve larval and post-larval yield under current and projected CO_2 conditions.

2. METHODS

2.1 G0 adults to G1 larvae and juveniles

Experimental methods comprising G0 broodstock conditioning through field sampling of G1 offspring at 9.5 months post-fertilization, are detailed in Friedman et al. (*in prep.*). Briefly, nine pedigreed families of *C. gigas* broodstock were obtained from Taylor Shellfish, Inc. (Shelton, WA). These were first-generation crosses originating from controlled pair-matings of wild Pacific oysters from Pipestem Inlet, British Columbia. Duplicate groups of 12 oysters per family were reproductively conditioned for 2 months under flow-through filtered seawater (FSW) at either ambient (1026$\pm$339 μatm) or elevated (1977$\pm$522 μatm) pCO_2 using standard hatchery methods. Following conditioning, 6 of the 9 families were selected for spawning based on the availability of a ripe male and female present in both treatment groups with ample, mature gametes. Oysters were strip-spawned into 1 μm-FSW buffered with 10% $NaCO_3$ (Ω_A: 2.5-3.0; pH: 8.15-8.4) at 25^{o}C. Gametes from each family (n=6) were fertilized in full factorial, minus

inbreds, for a total of 30 crosses. Fertilized embryos were reared under flow-through, 0.2 µm-filtered, buffered FSW at 25°C for 3 days until they reached the D-hinge veliger stage. Subsequently, equal numbers of larvae from each family were pooled into tanks receiving either ambient 0.2 µm-FSW at 25°C, or the same FSW adjusted to a target pH of 7.5 (Table 1), yielding 4 G1 treatment groups of larvae based on the combination of G0 broodstock pCO_2 exposure (elevated (H) or ambient (L)) and G1 larval pCO_2 exposure (elevated (H) or ambient (L)). Treatment groups were labeled in generational order (i.e. treatment HL = broodstock conditioned at 2000 µatm, larvae reared at 900 µatm (see Fig. 1). Larvae were reared under experimental conditions (Table 1) until they were large enough to be retained on a 236 µm screen and competent to settle (16 days). Larvae that did not reach competency by day 16 were removed from the experiment.

Table 1. Average experimental conditions (± standard deviation) during each phase of the study. ND = not determined.

Phase	Treat.	Temp. (°C)	Salinity (ppt)	pH	pCO_2	A_T	T_{CO2}	Ω Ar.	Ω Ca.	CO_3^{2-}
Broodstock condition	L	17.44±4.32	28.41±0.05	7.68±0.16	1026.13±339.25	2034.89±103.84	1967.80±85.95	1.13±0.48	1.76±0.75	70.48±29.77
	H	17.44±4.32	28.06±0.92	7.38±0.11	1977.86±522.31	1987.77±80.91	2007.65±90.39	0.53±0.04	0.83±0.07	33.07±2.75
Fertilization to Day 3	L	24.96±1.66	28.43±0.19	8.12±0.21	ND*	ND	ND	ND	ND	ND
	H	24.96±1.66	28.43±0.19	8.15±0.12	ND	ND	ND	ND	ND	ND
G1 Larval rearing Days 4-18	L	25.09±0.82	28.90±0.05	7.70±0.12	937.80±262.75	2051.40±13.87	1958.27±17.51	1.35±0.17	2.10±0.28	83.61±11.56
	H	25.09±0.82	28.95±0.12	7.50±0.09	1526.50±340.00	2065.11±31.10	2032.29±67.66	0.91±0.25	1.36±0.17	55.82±14.53
G2 Larval rearing	L	24.34±0.90	32.30±1.38	7.95±0.04	494.39±391.48	2128.16±20.25	1915.786±37.76	2.47±0.24	3.781±0.66	154.159±15.39
	H	24.41±1.03	32.17±1.34	7.49±0.04	1601.83±562.50	2136.77±28.21	2094.806±50.19	0.98±0.13	1.494±0.20	60.83±23.95

After 16 days in larval culture, settlement was induced in competent larvae using epinephrine following the methods of Coon et al. (1986). Newly-set juveniles were reared in the hatchery for 2 months, then placed in a common field nursery in Thorndyke Bay, North Hood Canal, WA, where they were held for an additional 2 months. Oysters were then deployed at 3 field sites: Sequim Bay (north Puget Sound), Totten Inlet (south Puget

Sound) and Thorndyke Bay (Hood Canal). Oysters were measured for growth (cm) before deployment into the field nursery (Day 91 post-fertilization) and again at Day 282, after 5 months at the 3 field sites.

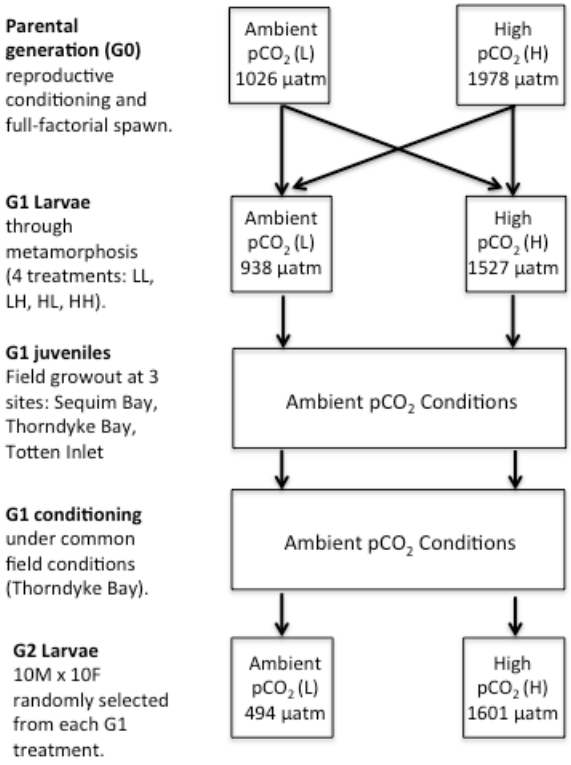

Figure 1. Flow chart of experimental design by generation and life-history stage.

2.2 G1 adults to G2 larvae

2.2.1 Field sampling of G1 adults

In December 2014, at approximately 21 months post-fertilization, oyster bags (n=99) from the four G1 treatments were retrieved from the Totten Inlet and Thorndyke Bay field sites at low tide. All live oysters were scraped of fouling organisms and blotted dry, after which individual measurements were taken of total wet weight (g). Digital

images were used to determine shell length and width (cm), and were analyzed using ImageJ (V.1.48). Oyster yield, a composite function of growth and survivorship (Langdon et al. 2003), was assessed as the total weight of live oysters per bag (kg live oyster weight bag^{-1}), standardized to a uniform stocking density of 50 oysters. Oysters corresponding to the upper, lower and middle 20th percentile in total weight for each bag were individually tagged (Floy Tags, Seattle, WA) and returned to Thorndyke Bay. The remaining oysters from each bag were shucked and measured for shell weight (g) and dry tissue weight (g). Dry weight measurements were obtained by oven drying oyster tissues at 60^{o}C for 96 hours to achieve a stable weight. Due to high field mortality among the Totten Inlet oysters and the need to retain sufficient broodstock to produce G2 larvae, lethal sampling for dry weight was not conducted for Totten oysters from treatment HL; this treatment group was therefore excluded from analyses of that data.

2.2.2. Genotyping G1 adults

G0 broodstock utilized in Phase I of our study consisted of a single sire and dam from each of 6 genetic families (see Friedman et al. *in prep.* for details but herein designated 1-6) conditioned at elevated and ambient pCO_2, respectively for a total of 24 individuals (Fig. 1). At the time of spawning, a small piece of mantle tissue was collected from each individual and preserved in 95% ethanol for subsequent DNA extraction and genotyping.

Genomic DNA was extracted from the ethanol-preserved mantle tissue samples from G0 broodstock using the Qiagen DNEasy® kit (Qiagen, Hilden, Germany) following manufacturer's protocol. Broodstock were genotyped at approximately 20 microsatellite loci to determine the most efficacious panel of markers for parentage

assignment. All microsatellite DNA markers were amplified individually in 10 µL PCR reactions containing the following components: 50-100ng of genomic DNA, 5x GoTaq buffer, 200 µM of dNTP's, 1.5 mM $MgCl_2$, 0.40 µM of locus-specific 5'-fluorescently labeled forward primer, 0.40 µM of reverse primer, and 0.5 U of GoTaq polymerase (Promega, Madison, WI). The following parameters were used for amplifications: 15 min at 95° C, 30 cycles of 30 s at 95° C, 45s annealing at 57° C, and 45s at 72° C. Following amplification, a final 10 min elongation step at 72° C was performed. Products were analyzed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). The resulting electropherograms were evaluated and sized using GeneMapper ver 4.0 (Applied Biosystems, Foster City, CA). We confirmed genotypic calls by independently amplifying and genotyping all parental samples multiple times.

The minimum number of loci which resulted in an assignment rate greater than 90% and a confidence of parent assignment greater than 95%, as calculated by simulation in the software package CERVUS (Kalinowski et al. 2007), were chosen for subsequent analysis.

In March 2015, we collected approximately 400 adult G1 oysters comprising the upper and lower 20th percentile in total weight (g) from treatments HL and LL within Thorndyke Bay and Totten Inlet, respectively. All oysters had been reared under the same ambient pCO_2 conditions as larvae. A sample of mantle tissue was non-lethally extracted from each oyster by relaxing oysters in a 7% $MgSO_4$ solution made with 1:1 mixture of ambient seawater and freshwater at room temperature for 2-6 hours. Tissues were preserved in ethanol, from which genomic DNA was extracted using Qiagen DNeasy kit (Qiagen, Hilden, Germany).

All offspring were genotyped at four microsatellite loci: ucdCg149, ucdCg171, ucdCg175 and Crgi236 (Li et al. 2003, Sekino et al. *unpublished data*), which were chosen based on the analysis described above, using the same conditions as described above. The final data set included genotypic data for 375 G1 adults, with less than 1% missing data. Parentage analysis was conducted in CERVUS (Kalinowski et al. 2007). Approximately 84% of offspring were assigned with 95% confidence, while 4% could only be assigned with 80% confidence, and 12% of offspring could not be assigned unambiguously. Parentage assignments were also tested in PAPA v.2.0 (Duchesne et al. 2002) and results were mostly concordant.

2.2.3 G2 spawning

Beginning in spring 2015, tagged, adult oysters from the four G1 treatment groups were allowed to reproductively condition under common, ambient conditions at Thorndyke Bay, Hood Canal, WA. Reproductive maturation was confirmed via visual inspection of gametes in a random selection of male and female oysters. In June 2015, oysters were transported to the NOAA Kenneth K. Chew Center for Shellfish Research (Manchester, WA). From each G1 group, 10 pairs of ripe males and females were selected at random, strip-spawned into ambient 1 μm-FSW at 25^{o}C, and gametes filtered of debris. Gametes from each female were counted using a Z2 Coulter Counter (Beckman Coulter Life Sciences, Indianapolis, IN), and fertilized for a total of 40 pair-matings in ambient FSW. Embryos from all pair-matings per G1 group were pooled at a density of 6 eggs/mL per female and quickly placed into replicate 4'' diameter downwelling silos (n=4) at ambient or elevated pCO_2 (494 or 1601 μatm; Table 1). After day 2, G2 larvae were fed a mixture of live algae (*Chaetoceros muelleri*, *Monochrysis lutheri*, *Pavlova* sp.,

and *Isochrysis galbana*) at a target density of 15,000 cells/mL. A subset of larvae from each silo were sampled in triplicate at days 2, 5, and 9 post-fertilization and preserved in ethanol for later examination. Larval samples were analyzed by light microscopy to estimate shell growth and survivorship, with digital images taken for analysis in ImageJ (V. 1.48). Day 2 larval samples were further inspected to estimate 'percent hatch', the proportion of fertilized embryos reaching the D-hinge veliger stage, and 'percent normal', the proportion of D-hinge veliger larvae with no visible shell deformities.

2.2.4 Carbonate chemistry manipulation

The elevated pCO_2 concentrations utilized in this experiment were obtained through direct manipulation of seawater pH via the bubbling of CO_2 gas into a header tank. The pH in each header tank was monitored by a DuraFET III pH probe (Honeywell, Morristown, NJ) connected to a Honeywell UDA2182 controller (Honeywell, Morristown, NJ), such that when pH rose above the desired set point, CO_2 gas would bubble through a solenoid valve (STC Valve, Palo Alto, CA) until the pH set point was reached. pH probes were calibrated weekly using Tris buffers and spectrophotometric pH per Dickson et al. (2007). Tank seawater pH, temperature, salinity and dissolved oxygen were monitored and recorded continuously using a YSI 5200A environmental probe (YSI, Yellow Springs, OH). Water samples from each tank were collected weekly and poisoned with 200 µl saturated $HgCl_2$ for later analysis of seawater pH, total alkalinity (A_T) and total dissolved CO_2 (T_{CO2}), per Barton et al. (Barton et al. 2012). An end point titration was used to measure A_T using a DL15 titrator (Mettler Toledo, Schwerzenbach, Switzerland). T_{CO2} was measured using a LI-COR LI-700 CO_2 detector (LI-COR, Lincoln, NE) as outlined in Dickson et al. (2007). Spectrophotometric pH was measured

on selected samples using SOP 6b of Dickson et al. (2007). The computer software CO_2calc (Lewis and Wallace 1998) was used to calculate seawater pCO_2, pH, and saturation state (Ω) of aragonite (Ω_A) and calcite (Ω_C) using the measured parameters of temperature, salinity, pH and A_T. Dissociation constants used in the CO_2calc program were obtained from Lueker et al. (2000), Dickson (1990), and Wanninkhof (1992). Precision and accuracy values were calculated by repeated measurements of certified reference materials (CRMs) supplied by Andrew Dickson's laboratory (Scripps Institution of Oceanography, La Jolla, CA).

2.3. Statistical analyses

Each mean value is expressed with its standard error of mean (mean $\pm$ SEM). For G1 adults, least squares regression and ANOVA were used to compare morphometric data among Site, G0 broodstock and G1 larval CO_2 treatment. The influence of Site, G0, and G1 CO_2 treatment on G1 survivorship was analyzed using generalized linear models with binomial error and "logit" link. We tested for density-dependent growth as a potential source of bias by regressing survival in each G1 oyster bag by the mean shell height of oysters in each bag. Parentage assignment data was used to tabulate the number of adult G1 oysters from each cross corresponding to either the upper or lower 20^{th} percentile in total weight sampled from both of treatments HL and LL within Thorndyke Bay and Totten Inlet, respectively. The distribution of the 6 parental families in each of these groups was analyzed using the chi-square test of independence, with separate analyses for sire and dam. We examined the influence of grandparental (G0), parental (G1) and acute (G2) CO_2 exposure, and their interactions, on shell growth in G2 larvae

using least squares regression and ANOVA. We used generalized linear models with binomial error and logit-link to examine acute and transgenerational effects of CO_2 exposure on G2 survivorship. Finally, we assessed the aforementioned effects on the percentage of normally-developed, D-hinge veliger larvae at Day 2, herein termed "percent hatch," using a generalized linear mixed model using *glmer* implemented in the R package 'lme4' (R Core Team 2017). We included silo as a random factor to account for overdispersion in the model. For all growth data, the Shapiro-Wilk test was used to confirm that data were normally distributed, and Bartlett's test was used to confirm that variances were homogeneous. Due to unequal variance, G1 dry weight data were log-transformed prior to analysis. Post-hoc comparisons of treatment means were performed using Tukey's Honestly Significant Difference test using *glht* implemented in the R package 'multcomp' (Hothorn et al. 2008). All statistical analyses were carried out in R (R Core Team 2017) at α=0.05.

3. RESULTS

Detailed statistical outputs, including F-statistics and effect magnitude, can be found in Supplemental Table 1. Any comparison listed herein, unless stated otherwise, was found to be statistically significant at α=0.05.

3.1 G1 Adults

Measurements were obtained for 2558 G1 adults in December 2014 (Totten Inlet n=841, Thorndyke n=1716). Total weight of G1 adults ranged from 3.62-192.27g, and was approximately twice as great among oysters from Totten Inlet (mean=111.29$\pm$1.01g)

than from Thorndyke Bay (mean=74.47±0.53g; Fig. 2). Across both sites, offspring of elevated pCO_2 parents exhibited ~6% lower total weight (mean=80.50±0.82g) than offspring of ambient CO_2 parents (mean=85.48±0.73g; Fig. 2), while larval CO_2 treatment did not influence adult total weight. Site-specific effects of parental and larval CO_2 treatment on G1 adult total weight followed similar trends (Fig. 2).

Dry weight (g) of G1 oysters was over twice as great on average in Totten Inlet oysters (7.249±0.37g) than Thorndyke oysters (3.133±0.05 g). Among Thorndyke Bay oysters, offspring of elevated pCO_2 parents exhibited reduced dry weight (2.942±0.06g) compared to offspring of ambient pCO_2 parents (3.317±0.7g; Fig. 2). While larval CO_2 treatment did not influence dry weight of Thorndyke Bay oysters, oysters from the LL treatment had greater dry weight than oysters from all other treatments, which were similar to one another (Fig. 2). Dry weight of Totten Inlet oysters did not differ across the three groups sampled.

Shell length in G1 adults was negatively influenced by exposure to elevated pCO_2 in parents and larvae. While Thorndyke oysters were larger in shell length (9.260±0.03 cm) than Totten Inlet oysters (8.927±0.05 cm), across both sites, exposure to elevated pCO_2 in either parents or larvae significantly reduced shell length in G1 adults compared to controls (treatment LL). While Thorndyke oysters followed this overall pattern, only HH oysters from Totten Inlet exhibited reduced shell height relative to controls, while treatments HL and LH were similar (Fig. 2).

Shell shape, as measured by the shell length-width ratio (LWR) varied with field site and was influenced by parental CO_2 exposure but not larval CO_2 treatment. Thorndyke Bay oysters were approximately 12% narrower on average (mean

LWR=1.645±0.005) than Totten Inlet oysters (1.467±0.008). Within and across both sites, offspring of H parents exhibited lower LWR than those of L parents (Fig. 2).

Survivorship from outplanting differed dramatically between field sites. Thorndyke Bay oysters exhibited 87.14±0.01% survival, while mean survivorship of Totten Inlet oysters was 43.61±0.02%. Within and across both sites, however, survivorship was unaffected by CO_2 treatment.

Oyster yield varied between sites, and was significantly higher in Thorndyke Bay oysters (3206.33±491.83 g) than Totten Inlet oysters (2380.26±813.99 g). Across both sites, yield was unaffected by parental or larval CO_2 exposure, or by their interaction. However, when assessed for Thorndyke Bay oysters individually, yield of LL oysters was over 13% greater (3410.47±504.37 g) than yield of HL oysters (3007.71±475.62 g).

We did not observe evidence of density dependent growth bias, in which per-bag survivorship and growth are inversely correlated. Rather, mean survivorship of G1 adults was positively correlated with shell length when assessed across both sites (ANOVA, r^2=0.10, F=10.69, p=0.0015). When assessed for each site independently, survivorship was not related to shell length.

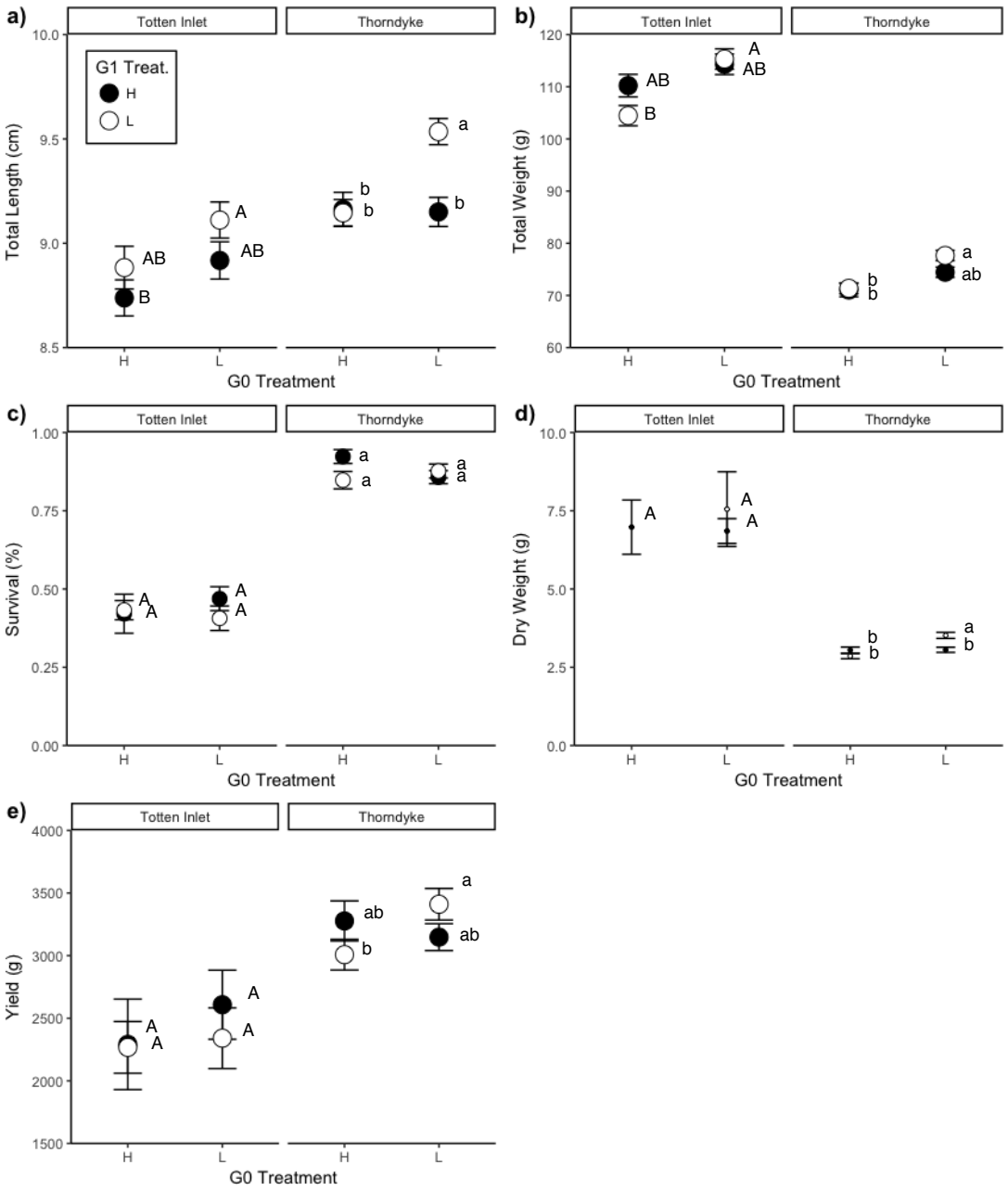

Figure 2. Performance (mean ± SE) of G1 adults at 21 months post-fertilization: A) total length (cm), B) total weight (g), C) survival, D) dry weight and E) yield. Shading corresponds to G1 larval CO_2 treatment: black = High pCO_2, white=Low pCO_2. Letters indicate significant differences based on Tukey post-hoc tests within each site. Upper and lower case letters indicate separate analyses.

3.2 Genotyping

Parentage was successfully assigned to 182 G1 oysters comprising the upper 20th percentile in total weight (g), and to 170 G1 oysters corresponding the lower 20th

percentile in total weight (g), from treatments HL and LL in Thorndyke Bay and Totten Inlet, respectively. Dam identity significantly influenced the genetic composition of offspring at both and both sites and at elevated and ambient pCO_2, while sire identity was only significant at ambient pCO_2 (Table 2). Analysis of Means for Proportion (ANOMP) for parental genotype among HL oysters revealed that at both sites, offspring of dam 4 were significantly more prevalent in the upper 20% pool, while offspring of dam 2 were significantly more prevalent in the lower 20% pool (Table 2). There were no significant differences in the proportion of offspring from any sire. Among Thorndyke Bay LL oysters, offspring of dam 1 were significantly more prevalent in the upper 20% pool, and offspring of dams 2 and 3 in the lower 20% pool. Offspring of sires 3 and 1 were significantly more prevalent in the lower 20% pool from Totten Inlet and Thorndyke Bay, respectively (Table 2).

Table 2. Results of chi-square test of independence and Analysis of Means for Proportion (ANOMP) of parental families (1-6) with significantly greater representation in the upper (U) or lower (L) 20% based on total weight (g) from treatments HL and LL at Totten Inlet and Thorndyke Bay, respectively. Data from each site/CO_2 is subdivided by dam (D) and sire (S) identity. A dash (-) signifies that no parental frequency was significantly greater than the mean, based on ANOMP. Significant *P*-values are in bold. α=0.05.

Site	Parent CO_2	Sex	N	df	χ^2	*P*-value	U	L
Totten Inlet	H	D	93	5	24.78	**0.0002**	4	2
		S	93	5	9.69	0.085	-	-
	L	D	90	5	11.812	**0.0375**	-	-
		S	90	5	17.056	**0.0044**	-	3
Thorndyke Bay	H	D	78	5	24.257	**0.0002**	4	2
		S	78	5	6.39	0.2701	-	-
	L	D	90	5	34.513	**<0.0001**	1	2,3
		S	90	5	12.487	**0.0287**	-	1

3.3 G2 Larvae

Percent hatch, or the proportion of fertilized embryos to properly develop into D-hinge veliger larvae, ranged from 61.84(±2.24)% in treatment LLH to 87.65 (±2.19)% in treatment LHL (Fig. 3). Hatching success was significantly reduced at elevated pCO_2, though the effects differed based on the transgenerational history of CO_2 exposure (Fig. 3; Table 3). Relative to controls (LLL), significant reductions in hatching success at elevated pCO_2 occurred when grandparents and parents both experienced elevated pCO_2 (HHH), and in the absence of transgenerational exposure to elevated pCO_2 (LLH) (Fig. 3). There was a significant grandparent-by-offspring interaction effect (Table 3) that appeared driven by the relatively low hatching success in treatment LLH (Fig. 3).

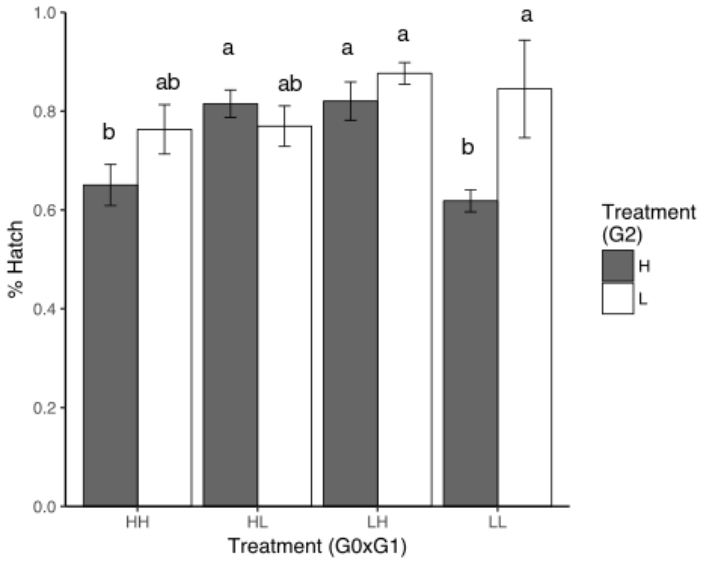

Figure 3. Percent hatch of G2 larvae (mean ±SE) at 48 hours post-fertilization. Transgenerational CO_2 exposure is shown on the x-axis as the combination of G0 and G1 treatments. G2 larval rearing environment is represented by dark shaded (H: 1601 µatm) or white (L: 494 µatm) bars. Letters indicate significant differences based on Tukey post-hoc tests.

Survivorship of the eight G2 larval groups through Day 9 ranged from 83.68(±3.07)% in treatment HHL to 45.94 (±2.60)% in treatment HHH (Fig. 4), and was significantly reduced at acute elevated pCO_2 (Fig. 4; Table 3). When survivorship was

directly compared across the eight G2 groups by incorporating G0, G1 and G2 CO_2 treatment, groups clustered predominantly based on their acute CO_2 exposure, with the notable exception of treatment HLH, which exhibited higher survival than the other G2 H groups and was statistically similar to the G2 L groups (Fig. 4). Neither parental nor grandparental CO_2 exposure influenced offspring survival (Table 3).

Table 3. Fixed effect results for Generalized Linear Mixed Model (GLMM) and Generalized Linear Model (GLM) constructed for G2 hatching and survivorship, respectively. Survival was modeled as a function of combined transgenerational CO_2 exposure, and again with grandparental, parental and acute CO_2 effects treated independently. Significant *P*-values are shown in bold.

Response	**Factor**	**df**	χ^2	***P* value**
Hatch	G0	1	2.983	0.084
	G1	1	0.459	0.498
	G2	1	7.625	**0.0058**
	G0 x G1	1	23.107	**<0.0001**
	G0 x G2	1	6.79	**0.009**
	G1 x G2	1	0.389	0.532
	G0 x G1 x G2	1	7.915	**0.0049**
Survival	G0 x G1 x G2	7	26.122	**0.0005**
Survival	G0	1	0.541	0.462
	G1	1	1.992	0.158
	G2	1	13.807	**0.0002**
	G0 x G1	1	0.973	0.324
	G0 x G2	1	0.008	0.927
	G1 x G2	1	2.348	0.125
	G0 x G1 x G2	1	2.625	0.105

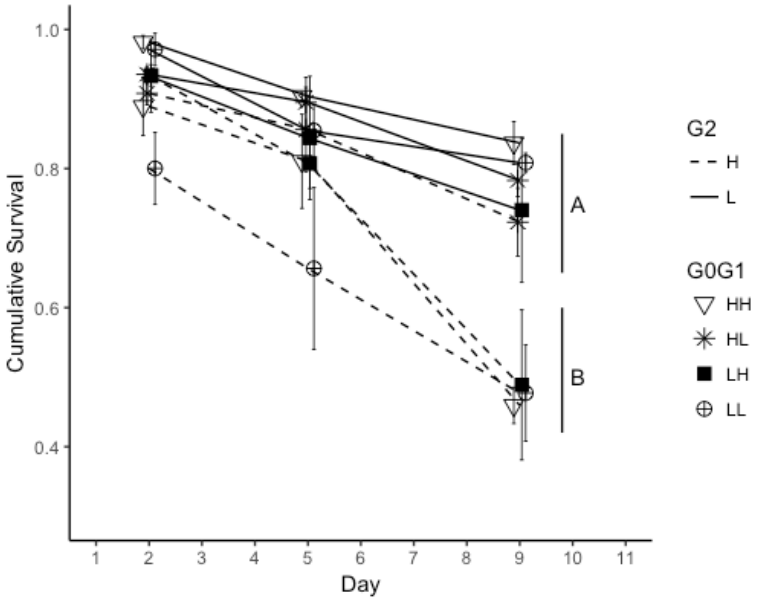

Figure 4. Cumulative survival of G2 larvae. Each symbol corresponds to a given G1 treatment group, denoted as the G0 parent and G1 larval CO_2 treatment, respectively. G2 larval rearing environment is denoted by a solid (L: 494 µatm) or dashed (H: 1601 µatm) line. Error bars correspond to SEM. Letters indicate significant differences based on Tukey post-hoc tests.

Shell length of G2 larvae at 48 hours post-fertilization was reduced at elevated pCO_2 (Fig. 5), and was influenced by parental CO_2 exposure (Table 4). Offspring from high CO_2 parents were on average smaller at hatching. While grandparental CO_2 exposure did not directly influence offspring size at hatching, we found a significant G0 x G2 interaction (Table 4), for which offspring reached larger sizes at hatching when reared at the same relative CO_2 treatment (ambient or elevated) as their grandparents (Fig. 5).

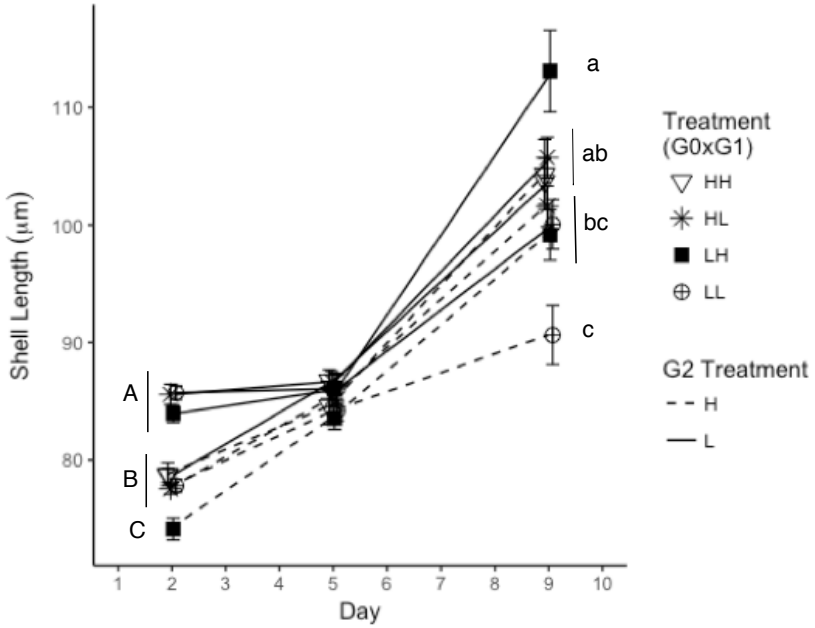

Figure 5. Mean (±SE) shell length of G2 larvae. Symbols represent transgenerational CO_2 exposure, denoted as the G0 parent and G1 larval CO_2 treatment, respectively. G2 larval treatment is denoted by a solid (L: 494 µatm) or dashed (H: 1601 µatm) line. Letters indicate significant differences based on Tukey post-hoc tests. Upper and lowercase letters indicate separate analyses.

At Days 5 and 9, larvae reared at elevated pCO_2 remained smaller than those reared under ambient conditions (Fig. 5). Parental CO_2 exposure influenced shell height at Day 9, however the direction of the effect differed from Day 2. Offspring whose parents experienced elevated pCO_2 as larvae were larger than offspring of ambient pCO_2 parents. Notably, larvae from treatment LHL were larger than most other treatment groups at Day 9 (Fig. 5). As in Day 2, we found a significant grandparent by offspring interaction (Table 4), which appeared driven by the relatively small size of LLH oysters, those with no history of transgenerational exposure to elevated pCO_2 prior to rearing at 1601 µatm.

Table 4. Selected models and Analysis of Variance (ANOVA) for larval shell length (μm) at three days of sampling depicting the effect of grandparental (G0), parental (G1) and acute (G2) CO_2 exposure. Significant *P*-values are shown in bold. α=0.05. Transgenerational CO_2 effects on shell length at Day 5 were non-significant and therefore omitted from the model.

Response	Factor	df	SS	MS	F Ratio	*P*-value
Shell Length (Day 2)						
	G0	1	9.466	9.466	0.247	0.620
	G1	1	1141.021	1141.021	29.717	**<.0001**
	G2	1	6093.702	6093.702	158.707	**<.0001**
	G0 x G1	1	0.359	0.359	0.009	0.923
	G0 x G2	1	889.264	889.264	23.160	**<.0001**
	G1 x G2	1	358.965	358.965	9.349	**0.002**
	G0 x G1 x G2	1	933.207	933.207	24.305	**<.0001**
	Residual	674	25878.882	38.400		
Shell Length (Day 5)						
	G2	1	430.442	430.442	7.546	**0.006**
	Residual	418	23844.237	57.044		
Shell Length (Day 9)						
	G0	1	512.582	512.582	3.456	0.064
	G1	1	1647.181	1647.181	11.107	**0.001**
	G2	1	2404.649	2404.649	16.214	**<.0001**
	G0 x G1	1	1444.786	1444.786	9.742	**0.002**
	G0 x G2	1	1304.871	1304.871	8.799	**0.003**
	G1 x G2	1	0.119	0.119	0.001	0.977
	G0 x G1 x G2	1	284.298	284.298	1.917	0.168
	Residual	239	35444.418	148.303		

4. DISCUSSION

In this study, we demonstrate that a long-lasting signature of parental exposure to elevated pCO_2 persists in G1 offspring through adulthood, with evidence of carryover into a subsequent G2 generation. G1 adults whose parents were conditioned at elevated pCO_2 exhibited reductions in both shell and somatic growth compared to offspring of control parents, and for all metrics analyzed in G1 adults, transgenerational plasticity

(TGP) did not buffer offspring from acute, negative effects of elevated pCO_2. Surprisingly, for some metrics, G2 larvae were most sensitive to acute elevated CO_2 in the absence of transgenerational exposure. The increased prevalence of particular genotypes in the pool of highest and lowest performing G1 oysters points to selection as an important force driving transgenerational CO_2 effects that warrants further investigation.

Our study builds upon work by Friedman et al. (*in prep.*), who first described transgenerational effects of elevated pCO_2 in larval and juvenile *C. gigas.* Compared to offspring of control parents, reproductive conditioning of G0 broodstock at 1977 μatm CO_2 was found to enhance developmental rates and survivorship in larvae reared under ambient (937 μatm) conditions (treatment HL), but resulted in synergistically negative effects on larval growth and survival when larvae were reared at 1527 μatm CO_2 (treatment HH). These trends remained largely the same in juveniles sampled at 1 and 3 months post-fertilization, respectively. Notably, however, when sampled at 9.5 months post-fertilization, offspring of elevated CO_2 parents had reduced shell weight and body weight compared to controls, with minor exceptions, regardless of larval CO_2 exposure (Friedman et al. *in prep.*). We found that these trends remained mostly consistent through 21 months post-fertilization, demonstrating that a long-lasting signature of parental exposure to elevated pCO_2 persists in G1 offspring through adulthood in spite of environmental stochasticity experienced during an additional year of field rearing. Control oysters from treatment LL exhibited the highest rates of shell growth, tissue growth, and per-bag yield at both Totten Inlet and Thorndyke Bay.

The contrast in performance between HL larvae/juveniles and HL adults underscores the importance of maintaining adequate experimental duration in transgenerational OA experiments, and suggests that previous studies documenting positive transgenerational effects of OA on invertebrate larvae and juveniles may in fact fail to capture long-term phenotypic trade-offs that arise as these animals reach reproductive maturity.

Several recent studies have demonstrated the influence of secondary stressors on transgenerational response to elevated CO_2. Parker et al. (2017) found that F1 "*transgen*" larvae, those spawned from parents conditioned at 856 μatm and reared at 856 μatm (similar to treatment HH in our study) developed faster and were 8% larger than offspring of control parents, but experienced significantly greater mortality in the presence of a secondary stressor such as elevated temperature, limited food, or lower salinity. Interestingly, these larvae exhibited higher Standard Metabolic Rate (SMR) than control larvae, a trait associated with increased acid-base regulation, protein synthesis and growth under OA conditions (Melzner et al. 2009, Lannig et al. 2010, Parker et al. 2012). While higher SMR and faster growth might benefit larvae exposed to elevated pCO_2 by reducing time to metamorphosis (Waldbusser et al. 2013), it was hypothesized that the additional energetic costs of a secondary stressor exceeded the larvae's physiological tolerance limits, resulting in mortality (Parker et al. 2017). Similarly, in the Olympia oyster, *Ostrea lurida*, transgenerational exposure to 1600 μatm CO_2 as a single stressor did not influence larval survival, but the additional stress of low dissolved oxygen (DO) resulted in significant larval mortalities (Wippel et al. *in prep.*). In our study, exposure of juvenile oysters to fluctuating estuarine conditions over a protracted field-rearing phase

may very well have interacted with transgenerational CO_2 exposure history to influence performance; relative performance of G1 groups varied to some degree between sites, particularly for dry weight and yield (Fig. 2), for which no CO_2 effects were observed at Totten Inlet. Puget Sound estuaries, like those in which our G1 oysters were matured, are characterized by boom-and-bust cycles of phytoplankton (Winter et al. 1975) in addition to typical estuarine fluctuations in temperature and salinity. Daily temperature data collected in the field during the G1 grow-out phase of our experiment (July 2013 – December 2014) did not reveal any anomalies (Friedman et al. *in prep*.), however we did not collect field data on ambient pH, salinity or phytoplankton biomass, which may have influenced growth and survival of G1 oysters at both sites. Indeed, survivorship was almost twice as great at Thorndyke Bay as it was at Totten Inlet (Fig. 2c) owing to a mortality event that occurred prior to sampling at 9.5 months (Friedman et al. *in prep.*). While there were no apparent density dependent growth effects on G1 oysters, environmental stress and mortality experienced at Totten Inlet may have selected for more stress-tolerant oysters based on both genotype and/or history of transgenerational CO_2 exposure.

Only a few recent studies to date have documented transgenerational plasticity (TGP) in response to elevated pCO_2 in organisms like molluscs and echinoderms with long generation times (e.g. Parker et al. 2012, 2015, Suckling et al. 2015, Thor and Dupont 2015, Ross et al. 2016), and evidence for beneficial TGP in response to elevated pCO_2, in which parental conditioning at elevated pCO_2 buffers offspring from acute negative effects (Foo and Byrne 2016), is limited for such organisms. In our study, beneficial TGP in G1 adults would manifest as higher performance of HH oysters than

LH oysters. For all G1 metrics examined, these two groups were either statistically similar, or HH performed worse than LH, suggesting that beneficial TGP in response to OA may not occur for *C. gigas* in one generation of carryover. Variation in performance of the eight G2 larval groups, however, suggests that acute response to elevated CO_2 is most severe in the absence of transgenerational exposure in either the grandparental or parental generation (*note* but not in both). Overall, G2 larvae from treatment LLH had the lowest metrics of hatching, size and survival.

In oysters, intraspecific variation in response to OA has been linked to heritable genetic variation (Parker et al. 2011, Frieder et al. 2017). While our experiment did not explicitly test for selection, our genotype data suggests that differential performance among crosses may have resulted in changes in genotype frequency of the surviving G1 oysters, with implications for G2 larvae. Using total weight (g) as our measure of performance, we observed significant differences in the prevalence of particular maternal and paternal lines among the highest and lowest performing G1 individuals, with differences based primarily on CO_2 exposure during conditioning, and to a lesser degree on field site. For example, at both sites, dams 4 and 1 produced disproportionately more of the largest and smallest offspring, respectively after conditioning at high CO_2 (Table 2).

Given our experimental design, these patterns may have arisen as early as fertilization and hatching in G1 larvae. For example, dam 4 exhibited the greatest percent hatch following conditioning at elevated pCO_2 (Friedman et al. *in prep.*). Although dam identity did not influence the genetic composition of HL larvae competent to settle at Day 16 (Friedman et al. *in prep.*), it is possible that offspring of this particular female

possessed physiological traits that contributed to faster growth post-settlement, such as increased feeding efficiency and lower metabolic cost of growth (Bayne 2004).

Although larvae from each G1 cross were pooled evenly into CO_2 treatments after hatching (Friedman et al. *in prep*.), G1 broodstock used to spawn G2 larvae were selected at random, and allele frequency changes in G1 oysters may well have resulted in reduced genetic diversity in G2 larvae, with selection for particular genotypes that were resilient following transgenerational CO_2 exposure, during larval rearing at elevated pCO_2, or following field outplanting. Hatchery rearing of *C. gigas* for aquaculture has been shown to result in rapid reductions in heterozygosity relative to wild stocks owing to genetic drift (Hedgecock & Sly, 1990). Similarly, for wild *C. gigas* stocks, effective population sizes are often quite low owing to sweepstakes reproduction, a mismatch between reproductive output and environmental conditions favoring fertilization, larval survival and recruitment (Hedgecock 1994). Distinguishing plasticity from evolutionary responses to transgenerational CO_2 exposure will be a critical goal for future studies to effectively model population response to OA (Donelson et al. 2017). For instance, maintaining crosses separately would allow for accurate estimations of within- and trans-generational plasticity, as well as genetic variance in performance (Donelson et al. 2017). Nevertheless, the fact that the genetic composition of offspring pools varied based on CO_2 exposure in both sires and dams suggests that OA may act as a potent source of environmental variability that affects the dynamics of larval recruitment and gene flow in wild and hatchery-propagated oyster populations.

Differences in the observed transgenerational effects of OA on marine invertebrates may be due to species-and population-specific responses to OA, and are

likely to be heavily dependent on experimental parameters. In echinoderms, whether transgenerational effects are positive or negative appears dependent on the duration of pre-exposure to elevated CO_2 in parents (Ross et al. 2016). For example, in the sea urchin *Sterechinus neumayeri,* parental conditioning at 1405 μatm CO_2 for 6 months resulted in smaller eggs and significantly greater larval mortality compared to control parents, but conditioning for 17 months alleviated those effects (Suckling et al. 2014). Similarly, for *S. droebachiensis,* 16 months of acclimation at 1217 μatm CO_2 was sufficient to reverse the negative effects on fecundity and larval survival observed after 4 months of pre-exposure (Dupont et al. 2013). In our study, G0 broodstock were conditioned at 1927 μatm for 2 months. It is plausible that extending this period of pre-exposure may have allowed for greater physiological acclimation, thereby improving offspring's ability to tolerate elevated pCO_2 conditions.

The pCO_2 levels chosen for experimentation may also influence the magnitude and direction of transgenerational effects. Experimentally elevated pCO_2 levels are often selected to simulate carbonate conditions projected to occur in the open ocean based on climate modeling scenarios. In the present study, we selected CO_2 treatment levels to compare performance under ambient estuarine conditions with a treatment level expected to occur in the open ocean within the next century (Caldeira and Wickett 2003, Orr et al. 2005, Doney et al. 2009b), but that is regularly experienced in NE Pacific estuaries during coastal upwelling events (Barton et al. 2012, Feely et al. 2012). Notably, in the first phase of our study, our ambient/low pCO_2 level was similar to, or higher than the elevated pCO_2 level used in other transgenerational OA studies (i.e. Parker et al. 2012, 2015, Miller et al. 2012), so our treatment pCO_2 level would likely represent an extreme

condition in such studies. These discrepancies make qualitative comparisons of results difficult, since the performance of our ambient pCO_2 group may be more reflective of an experimental group in other studies. It is plausible that positive transgenerational effects observed in other studies may be reversed if experimental carbonate conditions were more severe.

In conclusion, our results demonstrate that transgenerational effects of CO_2 exposure on the Pacific oyster will likely result in smaller, slower-growing individuals, which may affect ecosystem function in Pacific coast estuaries. Future research should be designed to explicitly disentangle genetic and non-genetic processes underlying these observed patterns. Because OA is expected to occur in tandem with increased sea-surface temperatures (Byrne 2011), future experimental design should also incorporate multiple stressors dual stressors, with experimental pCO_2 conditions that fluctuate on a diurnal basis as they do in nature (Cornwall et al. 2013). Indeed, recent evidence suggests that acute CO_2 effects are less severe when experienced on a fluctuating basis (Dufault et al. 2012). Such experimental considerations will allow for more robust measures of population's ability to respond to changing oceans.

5. REFERENCES

Barton, A., B. Hales, G. G. Waldbusser, C. Langdon, and R. A. Feely. 2012. The Pacific oyster, Crassostrea gigas, shows negative correlation to naturally elevated carbon dioxide levels: Implications for near-term ocean acidification effects. Limnology and Oceanography 57:698–710.

Barton, A., G. G. Waldbusser, R. A. Feely, S. B. Weisberg, J. A. Newton, B. Hales, S. Cudd, B. Eudeline, C. J. Langdon, I. Jefferds, T. King, A. Suhrbier, and K. McLaughlin. 2015. Impacts of coastal acidification on the Pacific Northwest shellfish industry and adaptation strategies implemented in response. Oceanography 28:146–159.

Byrne, M. 2011. Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. Oceanography and Marine Biology: An Annual Review 49:1–42.

Caldeira, K., and M. E. Wickett. 2003. Anthropogenic carbon and ocean pH. Nature.

Coon, S. L., D. B. Bonar, and R. M. Weiner. 1986. Chemical production of cultchless oyster spat using epinephrine and norepinephrine. Aquaculture 58:255–262.

Cornwall, C. E., C. D. Hepburn, C. M. McGraw, K. I. Currie, C. A. Pilditch, K. A. Hunter, P. W. Boyd, and C. L. Hurd. 2013. Diurnal fluctuations in seawater pH influence the response of a calcifying macroalga to ocean acidification. Proceedings of the Royal Society B: Biological Sciences.

Dickinson, G. H., A. V Ivanina, O. B. Matoo, H. O. Pörtner, G. Lannig, C. Bock, E. Beniash, and I. M. Sokolova. 2012. Interactive effects of salinity and elevated CO2 levels on juvenile eastern oysters, Crassostrea virginica. The Journal of Experimental Biology 215:29–43.

Dickson, A. G., C. L. Sabine, and J. R. Christian. 2007. Guide to best practices for ocean CO2 measurements. PICES Special Publication 3 3:191.

Doney, S. C., V. J. Fabry, R. A. Feely, and J. A. Kleypas. 2009a. Ocean Acidification: The Other

CO 2Problem. Annual Review of Marine Science 1:169–192.

Doney, S. C., V. J. Fabry, R. A. Feely, and J. A. Kleypas. 2009b. Ocean Acidification: The Other CO 2 Problem. Annual Review of Marine Science 1:169–192.

Duarte, C., J. M. Navarro, K. Acuña, R. Torres, P. H. Manríquez, M. A. Lardies, C. A. Vargas, N. A. Lagos, and V. Aguilera. 2014. Intraspecific Variability in the Response of the Edible Mussel Mytilus chilensis (Hupe) to Ocean Acidification. Estuaries and Coasts 38:590–598.

Duchesne, P., M. H. Godbout, and L. Bernatchez. 2002. PAPA (package for the analysis of parental allocation): A computer program for simulated and real parental allocation. Molecular Ecology Notes 2:191–193.

Dufault, A. M., V. R. Cumbo, T.-Y. Fan, and P. J. Edmunds. 2012. Effects of diurnally oscillating pCO2 on the calcification and survival of coral recruits. Proceedings of the Royal Society B: Biological Sciences.

Dupont, S., N. Dorey, M. Stumpp, F. Melzner, and M. Thorndyke. 2013. Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin Strongylocentrotus droebachiensis. Marine Biology 160:1835–1843.

Feely, R., T. Klinger, J. Newton, and M. Chadsey. 2012. Scientific Summary of Ocean Acidification in Washington State Marine Waters. Washington State Blue Ribbon Panel on Ocean Acidification. NOAA OAR Special Report.

Foo, S. A., and M. Byrne. 2016. Acclimatization and Adaptive Capacity of Marine Species in a Changing Ocean. Pages 69–116 Advances in Marine Biology.

Frieder, C. A., S. L. Applebaum, T. F. Pan, D. Hedgecock, and D. T. Manahan. 2017. Metabolic cost of calcification in bivalve larvae under experimental ocean acidification. ICES Journal of Marine Science 74:941–954.

Gavery, M. R., and S. B. Roberts. 2014. A context dependent role for DNA methylation in bivalves. Briefings in Functional Genomics 13:217–222.

Gazeau, F., L. M. Parker, S. Comeau, J. P. Gattuso, W. A. O'Connor, S. Martin, H. O. P??rtner,

and P. M. Ross. 2013. Impacts of ocean acidification on marine shelled molluscs. Marine Biology 160:2207–2245.

Gobler, C. J., and S. C. Talmage. 2013. Short- and long-term consequences of larval stage exposure to constantly and ephemerally elevated carbon dioxide for marine bivalve populations. Biogeosciences 10:2241–2253.

Hettinger, A., E. Sanford, T. M. Hill, E. A. Lenz, A. D. Russell, and B. Gaylord. 2013. Larval carry-over effects from ocean acidification persist in the natural environment. Global Change Biology 19:3317–3326.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biometric Journal 50: 346-363.

IPCC. 2013. IPCC Fifth Assessment Report (AR5). IPCC: s.10-12.

Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099–1106.

Kelly, M. W., and G. E. Hofmann. 2013. Adaptation and the physiology of ocean acidification. Functional Ecology 27:980–990.

Kroeker, K. J., R. L. Kordas, R. N. Crim, and G. G. Singh. 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecology Letters 13:1419–1434.

Kurihara, H. 2008. Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Marine Ecology Progress Series 378:275-284.

Kurihara, H., S. Kato, and A. Ishimatsu. 2007. Effects of increased seawater pCO_2 on early development of the oyster Crassostrea gigas. Aquatic Biology 1:91–98.

Langdon, C., F. Evans, D. Jacobson, and M. Blouin. 2003. Yields of cultured Pacific oysters Crassostrea gigas Thunberg improved after one generation of selection. Aquaculture 220:227–244.

Lannig, G., S. Eilers, H. O. Portner, I. M. Sokolova, and C. Bock. 2010. Impact of ocean acidification on energy metabolism of oyster, Crassostrea gigas - Changes in metabolic pathways and thermal response. Marine Drugs 8:2318–2339.

Lewis, E., and D. Wallace. 1998. Program developed for CO_2 system calculations. Oak Ridge National Laboratories 4735:1-21.

Marshall, D. J., and T. Uller. 2007. When is a maternal effect adaptive? Oikos 116: 1957-1963.

Melzner, F., M. A. Gutowska, M. Langenbuch, S. Dupont, M. Lucassen, M. C. Thorndyke, M. Bleich, and H.-O. Pörtner. 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6:2313–2331.

Miller, G. M., S.-A. Watson, J. M. Donelson, M. I. McCormick, and P. L. Munday. 2012. Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nature Climate Change 2:858–861.

Mousseau, T. A., and C. W. Fox. 1998. The adaptive significance of maternal effects. Trends in Ecology & Evolution 13:403–406.

Orr, J. C., V. J. Fabry, O. Aumont, L. Bopp, S. C. Doney, R. A. Feely, A. Gnanadesikan, N. Gruber, A. Ishida, F. Joos, R. M. Key, K. Lindsay, E. Maier-Reimer, R. Matear, P. Monfray, A. Mouchet, R. G. Najjar, G. K. Plattner, K. B. Rodgers, C. L. Sabine, J. L. Sarmiento, R. Schlitzer, R. D. Slater, I. J. Totterdell, M. F. Weirig, Y. Yamanaka, and A. Yool. 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681-686.

Parker, L. M., W. A. O'Connor, M. Byrne, R. A. Coleman, P. Virtue, M. Dove, M. Gibbs, L. Spohr, E. Scanes, and P. M. Ross. 2017. Adult exposure to ocean acidification is maladaptive for larvae of the Sydney rock oyster *Saccostrea glomerata* in the presence of multiple stressors. Biology Letters 13:20160798.

Parker, L. M., W. A. O'Connor, D. A. Raftos, H. O. Pörtner, and P. M. Ross. 2015. Persistence of

positive carryover effects in the oyster, Saccostrea glomerata, following transgenerational exposure to ocean acidification. PLoS ONE 10: e0132276

Parker, L. M., P. M. Ross, and W. A. O'Connor. 2011. Populations of the Sydney rock oyster, Saccostrea glomerata, vary in response to ocean acidification. Marine Biology 158:689–697.

Parker, L. M., P. M. Ross, W. A. O'Connor, L. Borysko, D. A. Raftos, and H. O. Pörtner. 2012. Adult exposure influences offspring response to ocean acidification in oysters. Global Change Biology 18:82–92.

Pespeni, M., E. Sanford, B. Gaylord, T. Hill, J. Hosfelt, H. K. Jaris, M. LaVigne, E. Lenz, A. Russell, M. Young, and S. Palumbi. 2013. Evolutionary change during experimental ocean acidification. Proceedings of the National Academy of Sciences 110:6937–6942.

Podolsky, R. D., and A. L. Moran. 2006. Integrating function across marine life cycles. Integrative and Comparative Biology 46:577–586.

R Core Team. 2017. R Development Core Team.

Razin, A., and A. D. Riggs. 1980. DNA methylation and gene function. Science 210:604–10.

Ross, P. M., L. Parker, and M. Byrne. 2016. Transgenerational responses of molluscs and echinoderms to changing ocean conditions. ICES Journal of Marine Science 73:537–549.

Salinas, S., S. C. Brown, M. Mangel, and S. B. Munch. 2013. Non-genetic inheritance and changing environments. Non-Genetic Inheritance 1:38–50.

Suckling, C. C., M. S. Clark, C. Beveridge, L. Brunner, A. D. Hughes, E. M. Harper, E. J. Cook, A. J. Davies, and L. S. Peck. 2014. Experimental influence of pH on the early life-stages of sea urchins II: increasing parental exposure times gives rise to different responses. Invertebrate Reproduction & Development 58:161–175.

Suckling, C. C., M. S. Clark, J. Richard, S. A. Morley, M. A. S. Thorne, E. M. Harper, and L. S. Peck. 2015. Adult acclimation to combined temperature and pH stressors significantly enhances reproductive outcomes compared to short-term exposures. Journal of Animal Ecology 84:773–784.

Thor, P., and S. Dupont. 2015. Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. Global Change Biology 21:2261–2271.

Timmins-Schiffman, E., M. J. O'Donnell, C. S. Friedman, and S. B. Roberts. 2013. Elevated pCO2 causes developmental delay in early larval Pacific oysters, Crassostrea gigas. Marine Biology 160:1973–1982.

US Department of Agriculture. 2013. 201 Census of Aquaculture.

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

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

Waldbusser, G. G., B. Hales, C. J. Langdon, B. A. Haley, P. Schrader, E. L. Brunner, M. W. Gray, C. A. Miller, I. Gimenez, and G. Hutchinson. 2015b. Ocean acidification has multiple modes of action on bivalve larvae. PLoS ONE 10.

Winter, D. F., K. Banse, and G. C. Anderson. 1975. The dynamics of phytoplankton blooms in puget sound a fjord in the Northwestern United States. Marine Biology 29:139–176.

Supplemental Table 1. ANOVA output for G1 adults sampled in December 2014. Effect magnitude is measured as η^2, the relative sum-of-squares for each effect. "Outcome" is based on Tukey post-hoc tests of significant effects. Site outcome is labeled as Thorndyke Bay (TH) or Totten Inlet (TO). *Dry weight (g) and Condition Index are only analyzed for TH oysters.

Response	Source	*df*	*SS*	MS	*F*-ratio	Pr. (> *F*)	η^2	Outcome
Shell height (G1)								
	Site	1	64.97	64.97	24.32	**< 0.00001**	0.01	TH > TO
	G0	1	30.36	30.36	11.36	**< 0.001**	0.00	L > H
	G1	1	33.29	33.29	12.46	**< 0.001**	0.00	L > H
	G0 x G1	1	18.58	18.58	6.95	**< 0.01**	0.00	LL > LH=HH=HL
	Residuals	3350	8951.2	2.67			0.98	
	Total	3354	9098.39	2.713				
Shell Width (G1)								
	Site	1	141.9	141.93	126.89	**< 0.00001**	0.04	TO > TH
	G0	1	8.2	8.18	7.31	**< 0.01**	0.00	H > L
	G1	1	21.4	21.386	19.12	**< 0.0001**	0.01	L > H
	G0 x G1	1	8.1	8.099	7.2408	**< 0.01**	0.00	HH=HL=LL > LH
	Residuals	3327	3721.4	1.119			0.95	
	Total	3331	3901	1.171				
Shell L/W (G1)								
	Site	1	19.52	19.52	287.72	**< 0.00001**	0.08	TH > TO
	G0	1	3.10	3.10	45.64	**< 0.00001**	0.01	L > H
	G1	1	0.04	0.04	0.53	0.47	0.00	
	G0 x G1	1	0.00	0.00	0.00	0.97	0.00	
	Residuals	3327	225.90	0.07			0.91	
	Total	3331	248.56	0.075				
Total weight (G1)								
	Site	1	877784	877784	1189.23	**< 0.00001**	0.26	TO > TH
	G0	1	25497	25497	34.54	**< 0.00001**	0.01	L > H
	G1	1	836	836	1.13	0.29	0.00	
	G0 x G1	1	3104	3104	4.20	**0.04**	0.00	LL > HL, HH; LH > HH
	Residuals	3351	2449581	731			0.73	
	Total	3355	3356802	1000.537				
*log(Dry weight) (G1)**								
	G0	1	0.44	0.44	17.48	**3.36E-05**	0.03	L > H
	G1	1	0.03	0.03	1.03	0.31	0.00	
	G0 x G1	1	0.23	0.23	9.06	**0.002728**	0.02	LL > LH=HH=HL
	Residuals	570	14.3458	0.03			0.95	
	Total	573	15.0397	0.0262472 95				

*Condition (G1)**								
	G0	1	5663	5663	7.3241	**< 0.01**	0.01	H > L
	G1	1	19	19	0.0245	0.876	0.00	
	G0 x G1	1	2226	2225.9	2.8789	0.09	0.00	
	Residuals	571	441468	773.1			0.98	
	Total	574	449376					
Survivorship (G1)								
	Site	1	1086.15	1086.15	270.365	**0**	0.03	TH > TO
	G0	1	0.06	0.06	0.015	0.903	0.00	
	G1	1	3.78	3.78	0.941	0.335	0.00	
	G0 x G1	1	0.96	0.96	0.2678	0.606	0.00	
	Residuals	94	35497.22	377.63			0.97	
	Total	98	36588.17	373.349				
Yield (G1)								
	Site	1	15913106	15913106	38.60	**< 0.00001**	0.29	TH > TO
	G0	1	707147	707147	1.72	0.19	0.01	
	G1	1	37090	37090	0.09	0.76	0.00	
	G0 x G1	1	288988	288988	0.70	0.40	0.01	
	Residuals	92	37927552	412256			0.69	
	Total	96	54873883	571602.95				