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Parental exposure of Eastern oysters (*Crassostrea virginica*) to elevated pCO_2 mitigates its negative effects on early larval shell growth and morphology

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PARENTAL EXPOSURE MODIFIES OA RESPONSE

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Abstract

Larvae of marine calcifying organisms are particularly vulnerable to the adverse effects of elevated pCO_2 on shell formation because of their rapid calcification rates, reduced capacity to isolate calcifying fluid from seawater, and use of more soluble polymorphs of calcium carbonate. However, parental exposure to elevated pCO_2 could benefit larval shell formation through transgenerational plastic responses. We examined the capacity of intergenerational exposure to mitigate the adverse effects of elevated pCO_2 on Eastern oyster (*Crassostrea virginica*) early larval shell growth, shell morphology, and survival. Adult oysters were exposed to control (572 ppm pCO_2) or elevated pCO_2 (2827 ppm pCO_2) conditions for 30 days during reproductive conditioning. Offspring from each parental treatment were produced using a partial North Carolina II cross design and grown under control and elevated pCO_2 conditions for three days. We found evidence of transgenerational plasticity in early larval shell growth and morphology, but not in survival, in response to the parental pCO_2 exposure. Larvae from parents exposed to elevated pCO_2 exhibited faster shell growth rates than larvae from control parents, with this effect being significantly larger when larvae were grown under elevated pCO_2 compared to control conditions. Parental exposure to elevated pCO_2, however, was insufficient to completely counteract the adverse effects of the prescribed elevated pCO_2 on early larval shell formation and survival. Nevertheless, these results suggest that oysters have some capacity to acclimate intergenerationally to ocean acidification.

Introduction

Ocean acidification caused by the absorption of increased atmospheric carbon dioxide threatens marine communities, particularly those dominated by calcifying organisms. Ocean

acidification decreases the pH and alters the carbonate chemistry of seawater, reducing the availability of carbonate ions and the associated calcium carbonate saturation state of seawater (Ω)—making it harder for many calcifying marine organisms to build their shells or skeletons (Orr et al. 2005). The precipitation of calcium carbonate is thermodynamically favored when Ω is greater than one, and dissolution is favored when Ω is less than one. Estuaries and coastal zones, regions where many calcifying organisms reside, already periodically experience Ω less than one (Waldbusser and Salisbury 2014; Wallace et al. 2014) and are thus highly vulnerable to future ocean acidification (Mackenzie et al. 2000; Melzner et al. 2013).

Early life stages of marine calcifying organisms are particularly vulnerable to the negative effects of elevated pCO_2 on calcification (Gazeau et al. 2013; Waldbusser et al. 2015a). Larvae form their first shells using amorphous calcium carbonate and/or aragonite, which are more soluble forms of calcium carbonate (Brečević and Nielsen 1989). Additionally, larvae exhibit decreased capacity to isolate their calcifying fluid from seawater (Waldbusser et al. 2013), which reduces their ability to control the chemistry at the site of calcification. Control of the carbonate chemistry at the site of calcification can influence an organism's calcification response to ocean acidification (Ries 2011; Liu et al. 2020). Furthermore, decreased Ω may alter larval energy budgets by increasing the energetic costs of calcification, potentially leading to developmental delays (Talmage and Gobler 2009). The effects of environmental stressors on larvae, including the adverse effects of elevated pCO_2 on larval calcification, may influence the performance of individuals in later life stages. These effects are referred to as 'carryover effects' (Gobler and Talmage 2013; O'Connor et al. 2014). For instance, abnormal early shell development could increase the risk of desiccation, predation, and natural mortality (Talmage and Gobler 2010; Gaylord et al. 2011). However, larvae of estuarine species, such as *Crassostrea*

hongkongensis (Hong Kong oyster), can exhibit resilience to the effects of elevated pCO_2 on shell growth (Lim et al. 2021a, b).

Transgenerational plasticity could influence early shell formation in larvae exposed to ocean acidification conditions and allow species to acclimate intergenerationally (i.e., adult to offspring) to the adverse effects of elevated pCO_2 on larval calcification. Organisms can respond to environmental stressors through plastic changes in phenotypes (e.g., morphology, gene expression, behavior; Chevin et al. 2013). Transgenerational plastic responses are passed from parent to offspring and interact with the offspring's environment to determine the offspring's phenotype (Salinas et al. 2013). Transgenerational plasticity occurs in response to various environmental stressors, including temperature, salinity, precipitation, and carbon dioxide-induced ocean acidification, and is taxonomically widespread (Salinas et al. 2013). Beneficial transgenerational plasticity in response to the effects of elevated pCO_2 on larval calcification could result from maternal provisioning and/or heritable epigenetic modifications. Maternal provisioning could increase energy reserves available to accommodate the increased energetic costs of calcification and survival under ocean acidification (Sunday et al. 2011; Swezey et al. 2020). Epigenetic modifications in gametes (Venkataraman et al. 2020) could be inherited by offspring and increase expression of genes involved in acid-base regulation (Parker et al. 2015) or calcification (e.g., genes identified by De Wit et al. 2018) and thus help mitigate the negative effects of acidification on offspring shell formation (e.g., Pespeni et al. 2013; Davies et al. 2016).

Transgenerational plasticity in bivalves may play a key role in the response of offspring to ocean acidification, but its impacts on larval performance vary by species. Parental exposure to elevated pCO_2 increases the resilience of *Saccostrea glomerata* (Sydney rock oyster) larvae by increasing their growth and development rates (Parker et al. 2012, 2015). Parental exposure also

85 increases the ability of *Mytilus edulis* (blue mussel) to form their first larval shells under elevated
86 pCO_2 (Thomsen et al. 2017) and improves juvenile growth and survival as seen in *C.*
87 *hongkongensis* (Lim et al. 2021b). In other species, however, parental exposure exacerbates the
88 adverse effects of ocean acidification by decreasing larval development rates, as observed for the
89 hard clam *Mercenaria mercenaria* and the bay scallop *Agropecten irradians* (Griffith and Gobler
90 2017). Understanding how parental exposure impacts larval responses to elevated pCO_2
91 increases our capacity to assess a species' potential for transgenerational acclimation to ocean
92 acidification but is still poorly understood across taxa.
93 Eastern oysters (*Crassostrea virginica*) are ecologically and economically important
94 calcifying organisms in estuarine environments of the northwest Atlantic that support diverse
95 benthic communities. Oysters have already experienced declines due to over-harvesting and
96 environmental degradation (e.g., Beck et al. 2011). Additionally, *C. virginica* exhibit negative
97 responses to intragenerational exposure to ocean acidification at all life-history stages, including
98 decreased growth (larvae: Gobler and Talmage 2014; juveniles: Beniash et al. 2010),
99 calcification (larvae: Miller et al. 2009; Talmage and Gobler 2009; juveniles: Dodd et al. 2015;
100 adults: Ries et al. 2009; Waldbusser et al. 2011), and survival (larvae: Talmage and Gobler 2009,
101 2012; juveniles: Beniash et al. 2010). But little work has examined how transgenerational
102 exposure to elevated pCO_2 could modify these responses (see Ross et al., 2016, for a review).
103 Clements et al. (2020) examined the effects of intergenerational exposure to elevated pCO_2 on
104 early shell formation (48-h) and survival in hatchery-reared *C. virginica* and found no significant
105 effects of parental exposure, but it is not known if wild-sourced *C. virginica* show the same
106 response. Understanding intergenerational responses of elevated pCO_2 exposure on early *C.*
107 *virginica* shell development will lay the foundation for future multi-generational (i.e., more than

two generations) studies to assess the potential for transgenerational acclimation to ocean acidification in Eastern oysters.

To investigate the potential for parental exposure to mitigate the negative effects of ocean acidification on early larval shell formation in wild-sourced Eastern oysters, we exposed adult oysters to control and elevated pCO_2 conditions for 30 days during reproductive conditioning and grew their larvae under control and elevated pCO_2 conditions for three days. Larval shell growth, shell morphology, and survival were measured to test the following hypotheses: (1) elevated pCO_2 negatively impacts shell growth, shell morphology, and survival of oyster larvae; (2) shell growth and shell morphology of larvae from parents exposed to elevated pCO_2 are less impacted by the adverse effects of elevated pCO_2; and (3) larvae from exposed parents exhibit higher survival rates than larvae from control parents under elevated pCO_2. We also examined the effects of parental exposure to elevated pCO_2 on egg size to assess whether maternal provisioning contributed to observations of transgenerational plasticity. Overall, this study aims to understand the potential effects of intergenerational exposure to elevated pCO_2 on larval shell growth and survival and gain insights into the mechanism(s) underlying these effects.

Methods

Adult oyster collection and exposure

Eighty adult specimens of *C. virginica* (mean ± SD shell length: 7.92 ± 1.80 cm) were collected from Barnstable Harbor, Barnstable, MA (41.714498, -70.333974) in late May 2018. Oysters were cleaned of epibionts, kept overnight in a 50-L sea-table, and labelled with tabs secured to their top valves using marine-safe cyanoacrylate (*Starbond* EM-2000 CA USA). Oysters were acclimated to laboratory conditions for one week under ambient conditions (mean

pCO_2 ± SD = 632 ± 64 ppm), then randomly assigned to control (572 ± 107 ppm) or elevated pCO_2 (2827 ± 360 ppm) conditions. The control condition was comparable to the first quartile of calculated pCO_2 for the collection site (median pCO_2 (IQR) = 777 (693) ppm). Collection site pCO_2 was calculated from measurements of dissolved inorganic carbon, total alkalinity, salinity, and temperature of monthly water samples taken at varying tidal timepoints (Figure S1.1). Since estuaries experience higher pCO_2 than average surface seawater of the open ocean (e.g., Waldbusser et al. 2011; Wallace et al. 2014), a pCO_2 level in the 500-600 ppm range was targeted to ensure that control treatment conditions (pCO_2, salinity, and resulting $\Omega_{aragonite}$) reflect those observed during summer months in Gulf of Maine estuaries (Waldbusser and Salisbury 2014). Likewise, the high pCO_2 treatment reflects elevated pCO_2 conditions that are already experienced over short time scales at the collection site (Figure S1.1a) and are expected to increase in duration for estuarine waters in the summertime (Melzner et al. 2013). This approach utilizes the concept of "extreme treatments," in which larger treatment differences increase the inferential and statistical power of the study design by reducing the influence of random noise and broadening the experimental domain (Whitlock and Schluter 2015).

Adult oysters were exposed to treatment conditions in 42-L tanks (10 oysters per tank, 4 tanks per treatment) for 30 days in a flow-through ocean acidification array that allows for control of temperature, salinity, and pCO_2. This timeframe produces differential epigenetic patterns in *C. virginica* gonad tissue in response to elevated pCO_2 (Venkataraman et al. 2020). The temperature of each tank was maintained at 20˚C and controlled using *Aqua Euro USA* model MC-1/4HP chillers (precision = 0.1˚C). Treatment tank salinity was maintained at 20, which is within the range of salinity that supports larval growth (Dekshenieks et al. 1993), by diluting incoming seawater from Broad Sound, Nahant, Massachusetts (42.416884, -70.907564)

with deionized water in a header tank. Flow rates of seawater to each tank were maintained at 40 mL min^{-1}, resulting in a tank seawater turnover time of 17.5 h. The pCO_2 of the control treatments was established by bubbling compressed ambient air into seawater. The pCO_2 of the elevated pCO_2 treatment was established by bubbling compressed air that was mixed with compressed carbon dioxide using solenoid-valve-controlled mass flow controllers (*Aalborg* Model GFC17, precision = 0.1 mL min^{-1}) at flow-rates proportional to the target pCO_2 condition. These flow-rates were then fine-tuned toward the target pCO_2 conditions based on measurements of the total alkalinity and dissolved inorganic carbon of the treatment seawater prior to the start of the experiment. Temperature, salinity, and pH_T (pH on total scale) of seawater in the experimental treatments were measured three times per week, which was sufficient to capture the mean values and the corresponding variances of the experimental treatment conditions resulting from natural variations in the composition of the source water. Seawater samples from each tank were collected during the first and third week of the exposure for analysis of dissolved inorganic carbon and total alkalinity. Adult oysters were fed *Shellfish Diet 1800* (*Reed Mariculture*) twice daily following established practices (Helm and Bourne 2004).

Larval crosses and exposure

At the end of the 30-day exposure, adults within each treatment were crossed using a partially factorial North Carolina II design (Lynch and Walsh 1998). Adult oysters were strip-spawned, with the goal of obtaining up to eight females and eight males per treatment. Oysters were sexed by examining a small sample of gametes under a microscope. The allocation of adults to specific crosses was randomized to eliminate selection bias. Eggs were rinsed into a weigh boat with control-treatment seawater and then filtered (*Pluriselect* filter stack 70 µm over 20 µm). Eggs on the 20 µm filter were rinsed into 150 mL of filtered seawater for crossing.

Additionally, unfertilized eggs were collected from each female and fixed with buffered 10% formalin (*Fisher* #SF100-4) for subsequent egg-size analysis. Sperm were rinsed with 1 mL of control-treatment seawater into a weigh boat, poured through a 10 µm filter, then added to jars containing eggs from designated females. Crosses were conducted under control conditions to ensure that any intergenerational effects measured were not a consequence of differences in the effects of elevated pCO_2 on fertilization (e.g., Barros et al. 2013). Eight control females, eight exposed females, five control males, and six exposed males were obtained for the fertilization crosses. Sperm were checked for mobility and only males with mobile sperm were crossed with ripe females, hence the difference in number of males for the control and exposed treatments. Each female was crossed with three males, except for one of the exposed females that was only crossed with a single male due to low egg counts. A total of 24 control and 22 exposed parent crosses were produced (Figure 1).

Two hours after fertilization, fertilization rates were quantified for each cross by counting the number of normally developing zygotes in a sample of 100. Zygotes were divided into six sterilized 250 mL jars filled with treatment seawater (3 control and 3 exposed) at a density of 1000 zygotes per jar (4 zygotes mL^{-1}), as estimated from fertilization rates, a density comparable to that occurring under hatchery conditions (Wallace et al. 2008). Larvae treatment seawater originated from 50-L header carboys that were treated with 500 mL of penicillin-streptomycin solution (*Sigma-Aldrich* Product No. P4458). Carboy water was bubbled with either ambient air (control treatment; mean $pCO_2 \pm SD = 665 \pm 74$ ppm) or ambient air mixed with carbon dioxide (elevated pCO_2 treatment; mean $pCO_2 \pm SD = 3340 \pm 241$ ppm) to produce the target pCO_2. Seawater samples from the header carboys were collected for measurement of pH_T and dissolved inorganic carbon before the start of the larvae exposure to assess drift throughout the experiment.

Jars were sealed without headspace and kept in a temperature-controlled room at 20˚C. No seawater changes were performed during the three-day larval exposure. Jars were stored in three sea-tables in a blocked design, such that one replicate control and one replicate elevated pCO_2 larvae jar for each cross were housed in each sea-table. At 48-h post-fertilization, larvae in each jar were fed 12 million cells of *Isochrysis* algae at a density of 40,000 cells mL^{-1}.

Commencing at ca. 68-h post-fertilization, each jar was measured for pH_T and filtered (*Pluriselect* filter stack 100 µm over 40 µm). Larvae on the 40 µm filter were rinsed with 10 mL of sterile filtered seawater into a 50-mL centrifuge tube. Filtering took several hours and was performed in blocks such that one replicate of every family in both larval treatments was filtered in a single block before the next block was filtered. Once all jars were filtered, 3.3 µL of neutral red dye was added to each sample and the sample was fixed with formalin (*Fisher* #500-00-00) to a concentration of 4% formaldehyde. After fixation, 10 mL of 10% sodium glycerophosphate was added to buffer the solution. Although larvae were filtered at different times, all larvae were fixed at the same time. Fixed larvae were refrigerated at 6˚C until analysis. Seawater samples from a subset of 10 larvae jars (5 control and 5 exposed) were collected for analysis of dissolved inorganic carbon following filtration of larvae.

Seawater carbonate chemistry

The temperature, salinity, and pH_T of adult treatment tanks, larvae jars, and larvae header carboys were measured using a glass thermometer (precision = 0.1˚C), a *YSI* 3200 conductivity probe (precision = 0.1 ppt), and an *Accumet* solid-state pH electrode (precision =1 mV) calibrated with pH 7.01 and pH 10.01 NBS buffers (for calibration slope) and Dickson seawater Certified Reference Material (for calibration intercept) to account for the effects of salinity on pH measurements (see Supplemental Materials Section 1).

Total alkalinity and dissolved inorganic carbon of seawater samples from adult and larvae exposures were measured with a VINDTA 3C system (*Marianda Corporation*). Total alkalinity was determined by open-cell potentiometric Gran titration (precision = 3 µmol kg-seawater^{-1}) and dissolved inorganic carbon was determined by coulometry (*UIC 5014;* precision = 3 µmol kg-seawater^{-1}). The precision of total alkalinity and dissolved inorganic carbon measurements was determined by repeated measurements of Dickson seawater Certified Reference Material. Samples were collected in 250 mL borosilicate glass bottles sealed with a vacuum-greased ground-glass stopper, poisoned with 100 µL saturated mercuric chloride solution, and refrigerated at 6˚C until analysis. Salinity, temperature, dissolved inorganic carbon, and total alkalinity were used to calculate pH_T, total alkalinity, $\Omega_{calcite,}$ $\Omega_{aragonite}$, carbonate ion concentration, bicarbonate ion concentration, dissolved carbon dioxide concentration, and pCO_2 of adult tank samples with CO_2SYS version 2.1 (Pierrot et al. 2011), using dissociation constants of carbonic acid (K_1, K_2) from Roy et al. (1993), a dissociation constant of hydrogen sulfate ($KHSO_4$) from Dickson (1990), and a boron concentration value from Lee et al. (2010). Carbonate parameters for larvae jars were calculated in the same way as for adult tanks, except pH_T was used instead of total alkalinity since pH_T was measured for each jar. The dissolved inorganic carbon of larvae jars that were not sampled for dissolved inorganic carbon analysis was estimated using the mean dissolved inorganic carbon measured for the subset of jars in each larvae treatment (control and exposed).

Egg and larvae morphology

The morphology of a subset of unfertilized eggs (30-32 eggs female^{-1}; except one exposed female, which only had 11 eggs measured due to filtering error) and larvae (n= 20 jar^{-1}; control parents: 72 jars per larvae treatment; exposed parents: 63 jars per larvae treatment) were

measured to characterize maternal provisioning and larval shell formation, respectively. Eggs and larvae were photographed with an Infinity Phase Contrast Trinocular Siedentopf compound microscope (*OMAX* 40X-2500X PLAN). The digital photographs were traced using GNU Image Manipulation Program (GIMP 2.10.8), and the resulting outlines were analyzed in CellProfiler (v3.1.5) to obtain egg diameter and egg eccentricity, larvae shell length, shell surface area, and shell perimeter (see Supplemental Materials Section 2). Eccentricity, which is equal to 0 for a circle and 1 for a line segment, was used to quantify egg shape.

The analysis of larval shell formation included only larvae that were D-stage (veliger) and lying flat in the field of view, such that the straight hinge was visible (Figure 2). Larvae with cilia extending beyond the shell margin were included, as were those with minor velum extrusion (Waldbusser et al. 2015b). Larvae without visible tissue in the shell were assumed dead and excluded from the analysis (ASTM 2012). Shell length was measured as the longest shell axis parallel to the hinge (Figure S2.1). The shape of larval shells was quantified as the ratio of perimeter to length. Larval growth was measured as the change in overall larvae shell size per day relative to egg size to account for differences in dam egg size (see Results). The change in size per day (ΔSize) was calculated for each larva as:

$$\text{eq. 1} \qquad \Delta\text{Size} = \frac{L - E}{D}$$

where L is the larva's shell length, E is the mean egg diameter for that larva's dam, and D is the larva's age in days.

The family percent change in shell growth and shell morphology for larvae grown in elevated pCO_2 compared to control conditions was calculated to measure inter-family variability in response to exposure. Percent change for individual families was calculated for each parameter (change in shell size, shell length, shell surface area, shell perimeter) as:

eq. 2 $$\%Change_{Family} = \frac{(X_{LarvalExposed} - X_{LarvalControl})}{X_{LarvalControl}} \times 100$$

where $X_{LarvalExposed}$ and $X_{LarvalControl}$ are the mean measurement of the parameter (X) of full sibling larvae grown in elevated pCO_2 and control conditions, respectively.

Larval survival

Larval survival was quantified for each jar by manually counting the larvae that took up neutral red dye (see Supplemental Materials Section 3). The estimation of the initial number of larvae mL^{-1} from fertilization rates resulted in small differences in the number of larvae added to each jar for different families, but similar numbers of larvae per jar within the same family. Therefore, the proportion of surviving larvae was quantified for each family (S_{Fam}) as:

eq. 3 $$S_{Fam} = \frac{S_{LarvalExposed}}{S_{LarvalControl}}$$

where $S_{LarvalExposed}$ and $S_{LarvalControl}$ are the mean number of surviving larvae from elevated pCO_2 and control jars, respectively. The family percent change in survival for larvae grown under elevated pCO_2 compared to control conditions was calculated in the same way as the family percent change in shell parameters (eq. 2).

Data analysis

Linear mixed-effects models were used to assess the effects of treatment on egg size and shape, larval shell growth, shell morphology, and survival. Parental treatment was treated as a categorical variable (control, exposed) and larvae treatment was treated as a continuous variable (jar $\Omega_{aragonite}$) to account for small differences in carbonate chemistry amongst replicate jars (see Results). Models were performed using the *lme4* (v.1.1-23; Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2017) packages in R (v.4.0.0; R Core Team 2020) using the graphical interface RStudio (v. 1.0.153). A step-down strategy with likelihood ratio tests was used to

290 determine the most parsimonious linear mixed model using the function *step()* from the *lmerTest*
291 package (Kuznetsova et al. 2017). Briefly, random effects were retained if the *p*-value based on
292 likelihood ratio tests was less than the α-level of 0.1; fixed effects were retained if the *p*-value
293 based on the calculated F statistic was less than the α-level of 0.05 (Kuznetsova et al. 2017).
294 Diagnostic Q-Q plots, residual-fitted plots, and autocorrelation plots were used to assess the
295 assumptions of normality, homoscedasticity, and autocorrelation, respectively. Appropriate
296 transformations were performed if data violated the assumption of normality (details below).
297 The $\Omega_{aragonite}$ of control and elevated pCO_2 treatment seawater from adult tanks and larvae
298 jars were compared using Welch's two-sample t-tests.

299 We tested the hypotheses that elevated pCO_2 impacted egg size and shape by testing the
300 response variables of egg diameter and eccentricity as a function of the fixed effects of dam
301 treatment (control, exposed) and dam length and the random effects of dam and acclimation tank.
302 Egg diameter ranged from 15.358 to 92.730 µm. Mature *C. virginica* eggs range in diameter
303 from 55 to 75 µm (Wallace et al. 2008). Eggs smaller or larger than this range were likely in
304 growing or degenerating stages of development, respectively (Lango-Reynoso et al. 2000).
305 Therefore, the data were subset using Tukey's method to identify and remove outliers that ranged
306 above and below 1.5 times the interquartile range. The resulting range in egg diameters used for
307 analysis was 49.953 to 75.970 µm. Importantly, running the analysis with outliers did not
308 qualitatively change the outcome. However, outliers were removed from the final analyses
309 because they were unlikely to have produced zygotes (Lango-Reynoso et al. 2000).
310 The effects of parental and larval exposure to elevated pCO_2 on larval shell growth and
311 shell morphology were assessed by evaluating the response variables larval change in size (i.e.,
312 ΔSize), shell length, √surface area, perimeter, and perimeter to length ratio. Surface area data

were square-root-transformed for analysis to meet the assumption of normality. The following full model was tested for each response variable (y):

$$\text{eq. 4} \quad y = \mu + ß_1 LExp + ß_2 PExp + ß_3 LExpPExp + (z_1 Dam) + z_2 Sire + z_3 Family + z_4 JarNumber + z_5 DamTank + z_6 SireTank + z_7 Table + \varepsilon$$

where *LExp* is a fixed effect of larvae jar $\Omega_{aragonite}$, *PExp* is a fixed effect of the parental treatment (control, exposed), *LExpPExp* is a fixed effect of the interaction between larvae jar $\Omega_{aragonite}$ and parental treatment, *Dam/Sire* is a random effect contributed by mother/father, *Family* is a random effect contributed by the specific parental cross, *JarNumber* is a random effect contributed by individual variations in larvae jars aside from jar $\Omega_{aragonite}$, *DamTank*/*SireTank* is a random effect contributed by individual variations in dam/sire treatment tank, *Table* is a random effect contributed by individual variations in the sea-tables in which the larvae jars were housed and also by the order in which the jars were processed during filtering, and ε is the residual error. Since the average egg size for each female was used to calculate change in larval shell size per day (eq. 1), *Dam* was excluded as a random effect for the model to assess growth to avoid violating the assumption of non-collinearity among explanatory variables. Post-hoc comparisons within larval treatments were conducted with two-sample t-tests using Bonferroni-adjusted *p*-critical values to understand better the effect of parental treatment on larval shell size and shell morphology (i.e., length, √surface area, perimeter, perimeter: length).

We tested the hypothesis that parental treatment influenced family survival by testing the response variable S_{Fam} as a function of the fixed effect of parental treatment and the random effects of *Dam*, *Sire*, and *DamTank/SireTank*. If larvae from exposed parents had relatively higher survival in elevated pCO_2 conditions than larvae from control parents, then $S_{Fam\text{-}ExposedParents} > S_{Fam\text{-}ControlParents}$. One-sample *t*-tests with Bonferroni-adjusted *p*-values were used to

examine the effect of larval treatment on survival within each parental treatment. If exposed larvae had relatively lower survival than control larvae, then family survival would be less than one (i.e., $S_{Fam} < 1$).

The relationship between larval growth rate and survivorship within the elevated pCO_2 larval treatments was examined by testing the hypothesis that family survival was influenced by mean family growth per day. If there is a tradeoff between growth and survival under elevated pCO_2 conditions, there should be an inverse relationship between family survival (i.e., S_{Fam}) and mean family growth per day, calculated as the average change in shell size per day (i.e., ΔSize) in the larval elevated pCO_2 treatment for each family. The response variable S_{Fam} was assessed as a function of mean family shell growth per day using a type II ordinary least squares (OLS) regression with 1000 permutations due to the error in the explanatory variable (Legendre 1998). Regressions were performed using the *lmodel2* package (Legendre and Oksanen 2018). Analyses for larvae with control and exposed parents were run separately because a significant interaction existed between parental and larval treatment on shell growth per day (see Results).

Results

Water chemistry

The $\Omega_{aragonite}$ of adult tank and larvae jar seawater differed significantly between control and elevated pCO_2 conditions (adult tank Welch's 2-sample *t*-test: $t = 12.33$, df = 7, *p*-value <0.0001; larvae jar Welch's 2-sample *t*-test: $t = 14.952$, df= 4.3, *p*-value< 0.0001). The elevated pCO_2 adult treatments exhibited higher total alkalinity and aragonite saturation state than the corresponding larval treatments due to partial dissolution of the adult shells under these conditions. However, the effects of these differences in alkalinity on seawater carbonate

chemistry were accounted for in the calculation of the saturation states of the treatments. There were moderate differences in carbonate chemistry amongst adult tanks of the same parent treatment, but they reflected ranges similar to those observed in the oysters' native waters (Table S1; Figure S1.1). Similarly, there were small differences in $\Omega_{aragonite}$ amongst jars of the same larval treatment. Based on larvae header and jar measurements, there was no significant drift in $\Omega_{aragonite}$ over the course of the three-day exposure for control (2-sample *t*-test: $t = 2.217$, df= 6, *p*-value >0.05) or exposed (2-sample *t*-test: $t = -0.013$, df= 5, *p*-value >0.05) larvae jars relative to the larvae header carboys.

Egg size and shape

There was no significant effect of parental treatment or dam length on egg size or shape. The most parsimonious models for egg diameter and eccentricity included only the random effect of dam. Dam explained 18.9% of the variance in egg diameter (variance ± SD dam: 4.331 ± 2.081) and 10.6% of the variance in egg eccentricity (variance ± SD dam: 0.001963 ± 0.0443). Because egg size varied significantly among dams, larval shell growth per day is a more accurate measure of larval growth compared to measurements of absolute larval size.

Larval shell growth and shell morphology

The best models for predicting early larval shell growth and shell morphology (length, √surface area, perimeter) included the main effects of parental treatment (control, exposed), larval treatment (jar $\Omega_{aragonite}$), and their interaction (Table 1). Larval exposure to elevated pCO_2 significantly decreased larval shell growth and shell morphology in every measured parameter (Figure 3). Notably, exposed larvae with exposed parents exhibited significantly higher overall growth rates and larger shells than larvae with control parents (2-sample *t*-tests: growth: $t = 14.10$, df = 1346, *p*-value <0.0001; shell length: $t = 8.18$,df = 1346, *p*-value <0.0001; shell

√surface area: t= 7.050, df = 1346, p-value <0.0001; shell perimeter: t= 7.190, df = 1346, p-value <0.0001), but this effect was small. Control larvae with exposed parents also exhibited significantly higher growth rates than control larvae with control parents (2-sample t-test: t= 10.01, df = 1348, p-value <0.0001). There were no significant differences in control larvae shell morphology based on parental treatment (2-sample t-tests: length: t= 1.47, df= 1348, p-value = 0.141; √surface area: t= 1.483, df = 1348, p-value= 0.138; perimeter: t= 1.237, df = 1348, p-value= 0.216). The mean shell growth per day for larvae with exposed parents was 1.15 and 5.88 times faster than larvae with control parents in control and elevated pCO_2 larval treatments, respectively. There was also high inter-family variability in the effects of larval environment on change in overall larval shell size and shell morphology (Figure 4). The mean family-specific reduction in overall size under larval elevated pCO_2 compared to larval control conditions was 87%, with nine families exhibiting mean reductions of over 100% (i.e., 3-day old larvae shells were smaller than the mean egg size of their dam). The family-specific reductions inshell length, surface area, and perimeter under larval elevated pCO_2 compared to larval control conditions ranged from 9 to 18%, 19 to 34%, and 10 to 19%, respectively.

Larval exposure to elevated pCO_2 also altered shell shape (Table 1). The shells of exposed larvae had decreased perimeter to length ratios, meaning that the shells were more elongated. Exposed larvae with exposed parents exhibited significantly lower perimeter to length ratios compared to exposed larvae with control parents (2-sample t-test: t= -3.936, df =1346, p-value <0.0001). There were no significant differences in shell shape of control larvae based on parent treatment (2-sample t-test: t= -0.47893, df = 1348, p-value >0.05).

Larval survival

Larval exposure to elevated pCO_2 significantly decreased survival within both control (one-sample *t*-test: t = -5.280, df = 23, p-value <0.001) and exposed (one-sample *t*-test: t= -4.021, df = 20, p-value <0.001) parental treatments. However, larval survival did not significantly vary with parental treatment. The best model to predict the proportion of surviving larvae included only the random effect of dam. Larval survival was highly variable among families (Figure 5). Six families exhibited increased survival under larval elevated pCO_2 conditions that ranged from 1 to 21% higher than survival under larval control conditions. The remaining 39 families exhibited decreases in survival under larval elevated pCO_2 conditions that ranged from 2 to 37% lower than survival under larval control conditions.

Although there was a positive trend, there was no statistically significant correlation between mean family shell growth per day and survivorship within the larval elevated pCO_2 treatment for three-day-old larvae with control (Type II OLS regression: p-value = 0.052, n = 24, R^2= 0.16, y= 0.876+ 0.045x) or exposed parents (Type II OLS regression: p-value = 0.104, n = 21, R^2= 0.13, y= 0.814 + 0.071x).

Discussion

This study illustrates the importance of considering parental environment and transgenerational plasticity in the response of *C. virginica* larvae to elevated pCO_2. The results demonstrate the following at three-days post-fertilization: (1) elevated pCO_2 significantly reduced shell growth, shell size (length, surface area, perimeter), and survival of oyster larvae; (2) larvae from exposed parents grew faster and had larger shells under elevated pCO_2 than larvae from control parents; and (3) parental exposure to elevated pCO_2 did not influence larval survival.

Effects of intergenerational exposure

Three-day-old Eastern oyster larvae of exposed parents exhibited evidence of beneficial transgenerational plasticity in early shell growth and shell morphology in response to elevated pCO_2. However, these positive carryover effects were not observed for larval survival. The shell growth rates and shell size (length, surface area, perimeter) of larvae with exposed parents were significantly higher than those of larvae with control parents under larval elevated pCO_2 conditions. These findings are similar to those of Lim et al. (2021b), who report that *C. hongkongensis* larvae with parents exposed to elevated pCO_2 ($\Omega_{aragonite}$ = 0.43) for 4 weeks during reproductive conditioning had significantly higher growth rates compared to larvae with control parents ($\Omega_{aragonite}$ = 1.55). Parker et al. (2012) also report similar findings for *S. glomerata* larvae with parents exposed to ocean acidification for 5 weeks , which exhibited significantly higher growth rates compared to larvae with control parents under both larval control ($\Omega_{aragonite}$ = 3.4) and ocean acidification conditions ($\Omega_{aragonite}$ = 1.9). The beneficial transgenerational plasticity observed in the present study suggests that *C. virginica* can acclimate intergenerationally to elevated pCO_2. It should be noted, however, that the effect sizes observed at three-days post-fertilization may not be biologically significant over longer timescales since it remains unclear whether the beneficial transgenerational plasticity observed would diminish, remain consistent, or increase across life-history stages (e.g., Lim et al. 2021b).

Interestingly, the positive effects of parental exposure to elevated pCO_2 on early shell growth and shell size (length, surface area, perimeter) were not significantly correlated with survival rates of exposed larvae. This finding suggests that intergenerational exposure to elevated *p*CO2 conferred no significant cost or benefit to larval survival within the framework of the experiment. This finding is consistent with prior studies on larval *C. virginica* (48-h post-fertilization; Clements et al. 2020) and *S. glomerata* (19 days post-fertilization; Parker et al.

2012). It remains unclear if the benefits of parental exposure on shell growth would continue to have no effect on larval survival beyond the duration of this experiment given the energetic costs of shell formation in undersaturated conditions, which potentially reduce energy budgets for maintenance and metamorphosis, as well as potential impacts on shell structure and function (Gaylord et al. 2011). However, it is possible that parental exposure could benefit *C. virginica* larval survival later in development, as seen for *M. edulis* seven days post-fertilization (control $\Omega_{aragonite}$ = 1.23; elevated pCO_2 $\Omega_{aragonite}$ = 0.36; Thomsen et al. 2017) and juvenile *C. hongkongensis* ($\Omega_{aragonite}$ = 1.55; $\Omega_{aragonite}$ = 0.43; Lim et al 2021b). Additionally, parental exposure could benefit fertilization rates under elevated pCO_2 conditions, an effect that was not assessed in this study since all crosses were conducted under control conditions.

The beneficial effects of transgenerational plasticity on early larval shell growth and shell morphology under elevated pCO_2 observed in this study on wild-sourced parents contrasts with the findings of Clements et al. (2020). The latter study, which exposed parents to experimental conditions for 29 days, found no significant effect of intergenerational exposure of hatchery-reared parents on shell length or shape of exposed larvae ($\Omega_{aragonite}$ = 0.4) at 48-h post-fertilization. Interpopulation variability has been observed for *S. glomerata,* in which wild-sourced populations exhibited beneficial transgenerational plasticity (as in this study) and hatchery-reared populations did not (as in Clements 2020) (Parker et al. 2012). These differences suggest that there may be population-level variation in the ability of *C. virginica* to acclimate intergenerationally to the negative effects of elevated pCO_2 on early shell formation.

The mechanism behind the observed beneficial transgenerational plasticity remains unclear. Parental exposure to elevated pCO_2 did not impact egg size, suggesting that maternal provisioning was not responsible for the increased shell growth of their larvae. Though notably,

egg size is not a perfect proxy for maternal provisioning. A plausible alternative hypothesis is that epigenetic inheritance mechanisms, such as DNA methylation and/or histone variants, are responsible for the observed effects (Eirin-Lopez and Putnam 2019). Eastern oyster gonad tissue exhibits changes in DNA methylation in response to ocean acidification, indicating the potential for heritability of methylation patterns (Venkataraman et al. 2020). However, the heritability of methylation patterns and the extent of transgenerational plasticity on *C. virginica* offspring phenotypes in response to ocean acidification (e.g., transient vs. enduring; Eirin-Lopez and Putnam 2019) is undetermined and requires further exploration. If transgenerational plasticity driven by epigenetic mechanisms is enduring, it could have positive carryover effects for shell production throughout larval development and settlement (Parker et al. 2015; Lim et al. 2021b) and result in effects of ocean acidification on larval shell formation that are less severe than those observed in intragenerational elevated pCO_2 exposure experiments (e.g., Miller et al. 2009; Talmage and Gobler 2009).

Effects of larval exposure

Despite the observed benefits of intergenerational exposure on early larval shell growth and shell morphology, larval exposure to elevated pCO_2 impaired larval shell formation and decreased survival, as has been previously documented in marine mollusks (e.g., Gazeau et al. 2013; Gobler and Talmage 2014; Kong et al. 2019). In contrast, Lim et al. (2021b) found *C. hongkongensis* larvae exposed to elevated pCO_2 exhibited faster development rates and increased settlement success compared to larvae in control conditions, suggesting certain estuarine species may exhibit increased capacity for adaptation to ocean acidification. Interestingly, Clements et al. (2020) found an increase in *C. virginica* larval survival under elevated pCO_2 ($\Omega_{aragonite}$ = 0.4) compared to control ($\Omega_{aragonite}$ = 1.0) conditions. Differential larval survival has been observed

for *M. edulis*, in which exposed larvae from parents sourced from lower-pH sites (as in Clements et al. 2020) exhibit higher survival than larvae from parents sourced from higher-pH sites (as in this study)(Thomsen et al. 2017).

Consistent with prior studies (e.g., Whitman Miller et al. 2009; Boulais et al. 2017), the present study did not find evidence of delays in early development of *C. virginica* larvae grown in undersaturated conditions. However, changes in shell morphology under elevated pCO_2 conditions could negatively impact shell function by altering the shell's ability to contain tissue and obtain food (Talmage and Gobler 2010). Additionally, changes in shell shape, as observed in this study, and disproportionate decreases in shell length and mass under elevated pCO_2, as observed in *C. gigas*, could alter larval distribution in the water column and impact dispersal, food availability, and predation rates (Stich and Lampert 1981; Sponaugle et al. 2002; Garcia Berdeal et al. 2006).

Inter-family variability

Genotypic differences contributed to larval oysters' responses to elevated pCO_2. Regardless of parental environment, families exhibited a range of responses to elevated pCO_2 in all measured shell parameters (shell growth per day, shell length, shell surface area, shell perimeter), as well as in larval survival. The significant random effects of family (for growth per day), dam (for shell length, shell surface area, shell perimeter, survival), and sire (for shell length, surface area, perimeter) show that certain *C. virginica* genotypes are more tolerant of elevated pCO_2 than others. In fact, the mean larval rate of shell growth for nine families (8 control parents, 1 exposed parents) under exposed larvae conditions was negative (i.e., larvae were smaller than mean dam egg size), while the remaining families exhibited an increase in size relative to egg size despite undersaturated conditions. Notably, there was a positive relationship

519 between shell growth rate and survival of exposed larvae, though it was not statistically
520 significant. This finding suggests that families with faster growing larvae are more tolerant of
521 elevated pCO_2, at least early in development, which contrasts with the inverse relationship
522 between growth and survival observed for *Haliotis rufescens* (red abalone; Swezey et al. 2020)
523 and *Strongylocentrotus purpuratus* (purple sea urchin; Garrett et al. 2020). Genotypes tolerant of
524 ocean acidification have also been observed for *C. gigas* (Frieder et al. 2017), *S. glomerata*
525 (Parker et al. 2011), and *M. mercenaria* (Waldbusser et al. 2010). Genotypes tolerant of elevated
526 pCO_2 evident in the present study could improve larval survival in future generations exposed to
527 ocean acidification. However, the positive carryover effects of genotypes tolerant of elevated
528 pCO_2 on larval survival may not extend beyond the first filial generation, as seen in *M. edulis*
529 populations (Thomsen et al. 2017). Nevertheless, the large inter-family variation in shell growth
530 per day and survival in the present study represents standing genetic variation that selection
531 could act on, potentially leading to adaptation of *C. virginica* populations to ocean acidification
532 conditions (Barrett and Hoekstra 2011).
533 Along with differences in source populations, the high inter-family variability in *C.*
534 *virginica* larval shell growth, shell morphology, and survival in the present study could explain
535 the contrasting results with Clements et al. (2020). Clements et al. (2020) found no significant
536 effect of intergenerational exposure on shell length or survival in *C. virginica* larvae grown
537 under control ($\Omega_{aragonite}$ = 1.0), intermediate ocean acidification ($\Omega_{aragonite}$ = 0.7), and high ocean
538 acidification ($\Omega_{aragonite}$ = 0.4) conditions at 48-h post-fertilization. Larvae in their study resulted
539 from gametes pooled from 4-6 individuals per sex (Clements et al. 2020). They were
540 consequently unable to account for inter-family variability in their statistical models. The present
541 study accounted for the effects of dam and sire by not pooling gametes, crossing each female

with multiple males, and growing offspring from each cross in control and elevated pCO_2 conditions. Therefore, the statistical models in the present study were better able to detect the effects of parental environment despite high inter-family variability.

Conclusion

In this study, we observed positive intergenerational effects of elevated pCO_2 exposure on early larval shell growth rate and shell morphology, without significant costs to survival. These findings suggest that oysters have the capacity to acclimate intergenerationally to ocean acidification (e.g., Parker et al. 2012, 2015; Lim et al. 2021b), though this capacity may vary by population (Clements et al. 2020). The high variability in rate of shell growth and survivorship among families also indicates the existence of genotypes tolerant of elevated pCO_2 that could be selected for under future ocean acidification conditions (Garrett et al. 2020). Collectively, these results suggest that selective breeding and/or parental exposure to elevated pCO_2 could improve production and increase the tolerance of Eastern oyster larvae to elevated pCO_2 in aquaculture and mariculture (Nell and Perkins 2005; Parker et al. 2011). Importantly, the seawater $\Omega_{aragonite}$ in both the control and elevated pCO_2 treatments in this study were less than one. Although consistent with the conditions that are experienced in Gulf of Maine estuaries (Waldbusser and Salisbury 2014), these conditions could influence the effect size of the observed transgenerational plasticity. Additional work is needed to understand the potential carryover effects of parental and larval exposure beyond early shell development, across different pCO_2 conditions, and in combination with other stressors (Przeslawski et al. 2015; Griffith and Gobler 2017). Further multi-generational and multi-stressor experiments that expand on this study will improve our understanding of larval responses to oceanic change and enable better prediction of carbon dioxide-induced changes in marine communities.

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Author Contributions

The experiment was designed by AD-W, KEL, and JBR. AD-W conducted previous ocean acidification exposures with Eastern oysters used for planning seawater chemistry targets and aided in planning of the pair-mated cross design; KEL designed the pair-mated cross and conducted a pilot study in 2016; JBR designed the ocean acidification and gas-mixing systems and the plan for analyzing seawater chemistry. Seawater collections from the field were performed by AD-W and EMM. The methods for phenotyping and analyzing larval shell properties and larval survival were developed by EMM, AD-W, KEL, and JBR. Personnel were trained by EMM, AD-W, KEL, and JBR. Data were collected by EMM, ADW, FDT, CC, and KEL; JBR supervised collection and management of seawater chemistry data. Statistical analyses were developed by EMM with assistance from KEL and JBR. Data visualizations were created by EMM with input from KEL and JBR; KEL created the visualization of the partial North Carolina II cross design. Funding was obtained by KEL and JBR. The manuscript was written by EMM. All authors contributed to editing the final version of the manuscript and approve of its submission.

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Tables

Table 1. Summary of the most parsimonious linear mixed effects models, as determined by model selection using the *step*() function with the *lmerTest* package, that predict larval change in shell size and shell morphology.

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	Fixed effect	Coefficient ± SE	df	*t*-value	*p*-value	Random effect	Variance ± SD
	Intercept	0.352 ± 0.188	30.083	1.869	0.0714	Family	0.447 ± 0.669
ΔSize	Parental treatment	-1.043 ± 0.233	63.721	-4.479	**<0.0001**	Jar Number	0.206 ± 0.454
R^2=0.82	Larvae $\Omega_{aragonite}$	6.838 ± 0.201	220.864	33.958	**<0.0001**	Sea-table	0.019 ± 0.139
	Interaction	0.761 ± 0.273	220.311	2.782	**0.0059**	Residual	0.856 ± 0.925
	Intercept	63.047 ± 0.528	18.544	119.419	**<0.0001**	Dam	0.671 ± 0.819
	Parental treatment	-1.651 ± 0.656	24.520	-2.518	**0.0187**	Sire	0.326 ± 0.571
Length	Larvae $\Omega_{aragonite}$	20.493 ± 0.608	240.574	33.718	**<0.0001**	Jar Number	1.897 ± 1.377
R^2=0.80	Interaction	2.260 ± 0.825	240.002	2.739	**0.0066**	Sea-table	0.173 ± 0.416
						Residual	7.704 ± 2.776
	Intercept	51.330 ± 0.484	11.567	106.134	**<0.0001**	Dam	0.479 ± 0.692
	Parental treatment	-1.068 ± 0.546	24.587	-1.956	0.0619	Sire	0.213 ± 0.462
$\sqrt{\textbf{Area}}$	Larvae $\Omega_{aragonite}$	18.269 ± 0.509	240.309	35.922	**<0.0001**	Jar Number	1.369 ± 1.170
R^2=0.83	Interaction	1.391 ± 0.690	239.982	2.015	**0.0450**	Sea-table	0.241 ± 0.491
						Residual	4.983 ± 2.232
	Intercept	194.747 ± 1.761	14.931	110.572	**<0.0001**	Dam	7.109 ± 2.666
	Parental treatment	-4.146 ± 2.095	24.434	-1.979	0.0592	Sire	3.138 ± 1.772
Perimeter	Larvae $\Omega_{aragonite}$	69.692 ± 1.940	240.314	35.932	**<0.0001**	Jar Number	20.023 ± 4.475
R^2=0.83	Interaction	5.559 ± 2.633	239.886	2.111	**0.0358**	Sea-table	2.519 ± 1.587
						Residual	71.329 ± 8.446
Shape	Intercept	3.092 ± 0.004	18.800	673.548	**<0.0001**	Dam	$5.56x10^{-5}$ ± 0.007
(Perimeter:	Parental treatment	0.015 ± 0.006	31.734	2.673	**0.0118**	Jar Number	$1.21x10^{-4}$ ± 0.011
Length)	Larvae $\Omega_{aragonite}$	0.079 ± 0.007	251.247	11.512	**<0.0001**	Sea-table	$1.24x10^{-5}$ ± 0.004
R^2=0.18	Interaction	-0.023 ± 0.009	250.328	-2.420	**0.0162**	Residual	$2.19x10^{-3}$ ± 0.047

Figure Legends

Figure 1. Diagram of the partial North Carolina II cross experimental design. Control parents and control larvae jars are indicated in blue; exposed parents and exposed larvae jars are indicated in pink.

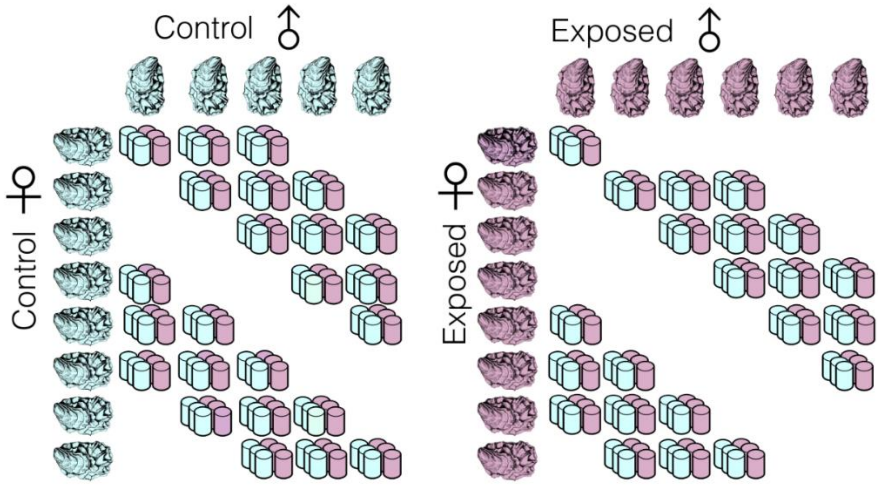
Figure 2. Representative images of larval shells from parental and larval treatments. Scale bars are 25µm.

Figure 3. Shell growth (a; 'ΔSize'), shell length (b), square root of shell surface area (c), and shell perimeter (d) of three-day-old Eastern oyster larvae grown at different $\Omega_{aragonite}$ from parents exposed to control (blue) or elevated pCO_2 (pink) conditions. Points represent individual larvae. Regressions represent the most parsimonious linear mixed-effects models for the observed parameters; shaded areas represent the 95% confidence intervals for the models.

Figure 4. Boxplots of control (blue) and exposed (pink) larval change in shell size (a), shell length (b), shell surface area (c), and shell perimeter (d) from control (blue panel) and elevated pCO_2 (pink panel) parental crosses. Dashed lines indicate the mean value for control (blue) and exposed (pink) larvae from control parent crosses.

Figure 5. Reaction norms of family survival in control and elevated pCO_2 conditions for *C. virginica* larvae with control and exposed parents. Each line represents a unique family; points represent the mean (±SE) number of surviving larvae within each treatment for each family.

813 **Figure 1.**



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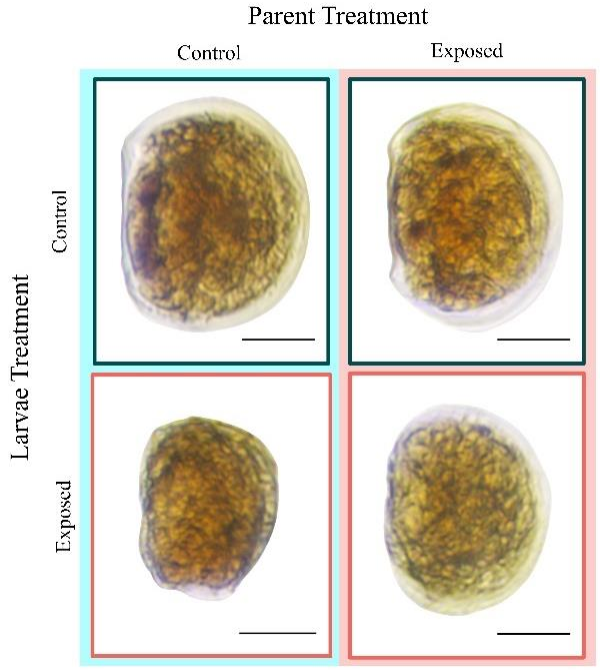
816 **Figure 2.**



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820 **Figure 3.**

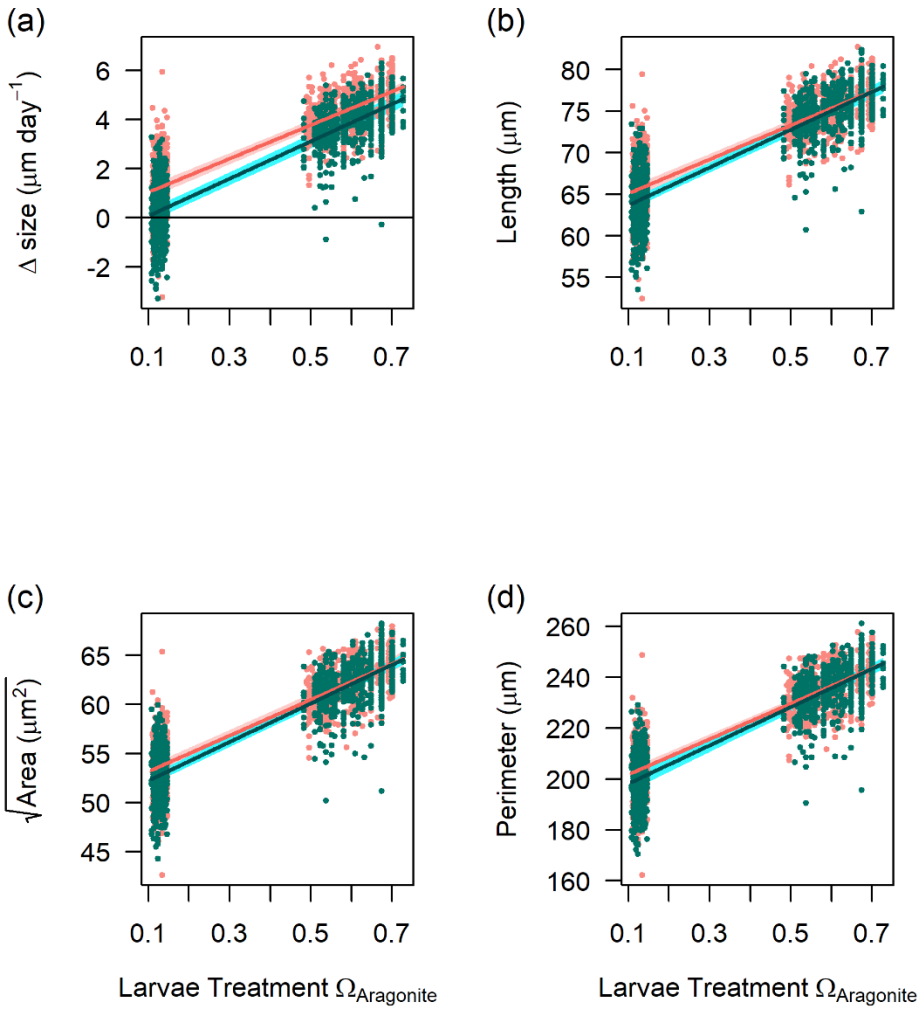

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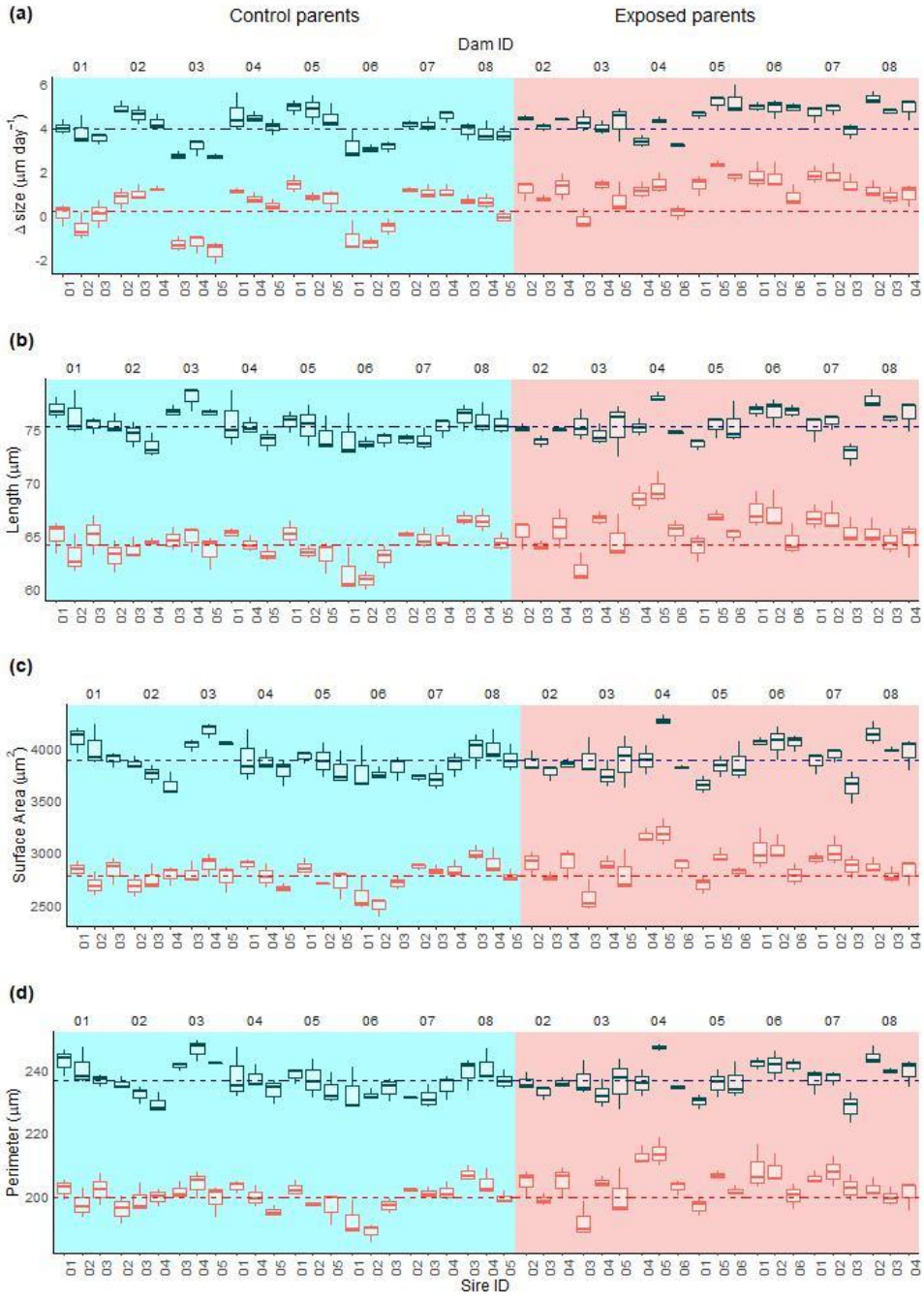
Figure 4.


(a)
Control parents
Exposed parents
Dam ID
Δ size (µm day⁻¹)
(b)
Length (µm)
(c)
Surface Area (µm²)
(d)
Perimeter (µm)
Sire ID

825 **Figure 5.**

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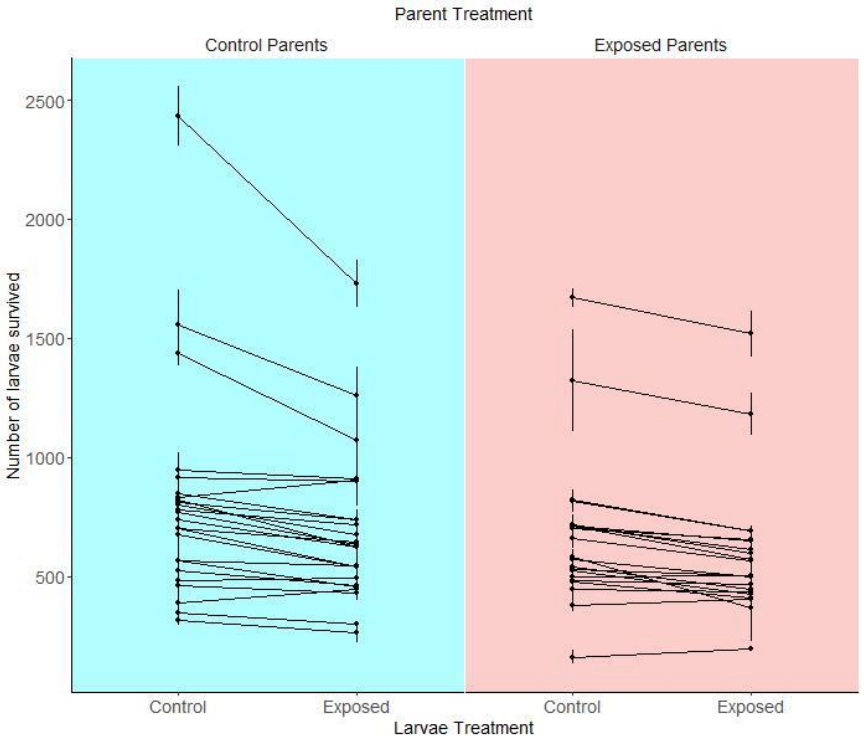

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