***Elevated TA conditions influence juvenile oyster growth when combined with a lower salinity***

1,2Saley, Alisha M., 2Kimball, Zoe & 1,2Brian Gaylord

1 Bodega Marine Laboratory, University of California Davis

2 Dept. of Ecology and Evolution, University of California at Davis

***Abstract—***

Response trajectory—do effects change over time, bigger/small and ibn what direction

Reasonable expectations; which one plays out

***Introduction—*** Coastal estuaries exhibit variability in the seawater carbonate system, with implications for calcifying inhabitants. For example, biochemical processing and river inflows can cause gradients of total alkalinity (TA) over small distances (cite). Estuarine TA conditions are temporally complex, where conditions can change abruptly and persist for weeks to months, requiring sessile calcifiers to new conform to new conditions. Given the multi-faceted extent to which external seawater conditions dictate performance for marine calcifiers, investigating how responses to such variation in TA change through time, deserves specific attention.

Substantial effort has documented how calcifier growth can be disrupted by perturbations to the carbonate system. Some have emphasized the temporal nature of perturbed conditions, both within and across life stages, finding that the nature of exposure (statis vs fluctuating) and the life-stage at exposure (X vs X) can influence future growth performance (cite). A gap remains in our understanding of the degree to which responses may vary, between the initial period following exposure and a latent period, after an individual has experienced the new seawater condition for multiple weeks’ time. Following exposure to new conditions, many calcifiers conform their internal fluids to match external conditions, creating physiological trade-offs between investing energy into maintenance versus growth (shell or tissue). As such, overall patterns of net growth to variable conditions could look similar in individuals that, in fact, exhibited very different growth patterns through time.

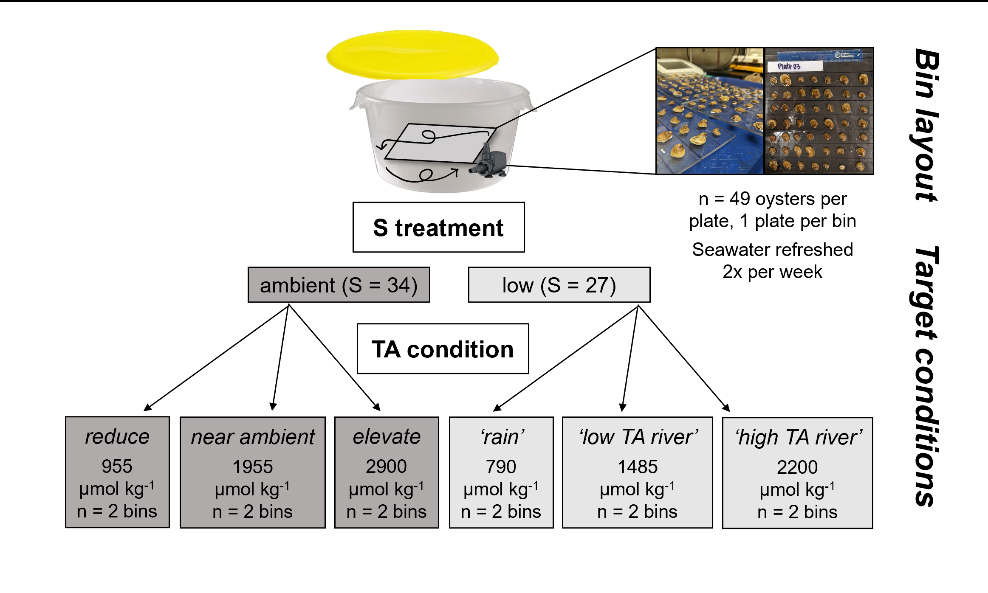
Exploring how growth rate responds to altered TA conditions through time is a natural complement to prior work. Others have documented disrupted growth in many calcifiers following abrupt shifts to carbonate system conditions (cite), with substantial attention devoted to understanding the consequences of human-derived ocean acidification (for review see Gazeau et al 2013). Drastic drops in salinity also have consequences on growth (cite), though, diluted TA may have interacted with lower salinity. Observed declines in growth can signify metabolic downregulation (cite) and an unmet increase in energetic demand (cite), which could result in trade-offs between shell and tissue growth (cite). What remains unexplored is how growth may change over time in response to variable TA conditions, specifically in calcifiers known to experience, and tolerate, a wide range of conditions.

In estuaries, oysters have significant economic and ecological value to coastline habitats and communities. As oysters naturally form reefs, they provide habitat for other estuarine species (cite) and can protect shoreline from X (cite). Commercially, they are be grown and harvested for consumption, economically contributing to a growing human shellfish diet (cite). As such, emphasis is often placed on both growth of the shell and of tissue mass. Juvenile growth in particular can be sensitive to abrupt changes in conditions (cite), which, could result in trade-offs between shell or tissue growth (cite).

Here we explore the how influence of TA condition on surface area shell growth can vary between initial and latent periods in the juvenile Eastern oyster (*Crassostrea virginica*), depending on the salinity. We quantified incremental surface area growth of oyster valves across two time periods as a function of TA condition and accounted for the effect of oyster size at the start of the period, separately, in ambient and low salinity treatments. We did not observe differences in surface area growth initially among TA treatments, in either salinity. Growth rate patterns changed slightly in the latent period, where oysters in low salinity treatments combined with elevated TA exhibited higher growth rates than those in TA conditions simulating dilution with DI. Additionally, growth rates were lower in the latent period than the initial period, in all TA conditions. Given the known shifts between periods of energy assimilation and storage, shell growth, or tissue growth in oysters, we also compared average oyster shell thickness (shell mass per area) and condition index (tissue mass per shell mass) as a function of TA conditions. In ambient and salinity treatments, we did not see any trade-offs to tissue mass or shell thickness, as neither oyster shell thickness nor condition index varied as a function of TA condition. Examinations such as these lend insights into oysters may respond to abrupt changes in estuarine conditions through time.

***Methods—***

Fig. 1



**Study species—** The Eastern oyster, *Crassostrea virginica*, forms native, subtidal reefs along eastern shores of North America (Gulf of St Lawrence to the Gulf of Mexico) (cite) but has been introduced and grown commercially elsewhere (cite), due to a wide salinity and thermal tolerance. We received juvenile oysters from an oyster farm in Tomales Bay, CA (Hog Island Oyster Company; coordinates) ~ 6 months after their arrival from X stock location. On 22 July 2022, oysters were transported in chilled seawater (~60 min) to the UC-Davis’ Bodega Marine Laboratory (BML; Bodega Bay, CA), where they were placed on flow-through laboratory seawater sourced from the adjacent coastal ocean. We supplemented lab seawater with continuous bubbling and slow-release mixed algal diet (X% of their wet mass) every other day, over the course of 30 days acclimation to lab conditions. Three days prior to the start of the experiment we adhered the oysters (left valve) to plastic plates using X marine epoxy (n = 49 per plate, n = 12 plates) following X et al (cite year). Plates were then returned to seawater acclimation conditions and visually assessed daily for open feeding activity.

**Oyster growth experiment—** We tracked shell growth rates of oysters exposed to prescribed seawater conditions along a response trajectory to separate initial and later effects of TA condition from overall patterns of shell and tissue growth (see *Experimental conditions*, Fig. 1). On three occasions along the trajectory (day 0 (initial), 18, 36), we took photos and used ImageJ software (v.X) to analyze valves surface area (with a scale bar). Then, we quantified surface area growth over the early () and later () increment separately, as the increase in valve area per day (mm2 d-1) relative to the area of the valve at the start of the increment. We calculated shell thickness and condition index at the end of the experiment, needing to first sacrifice oysters and dry tissue separate from the shell at 60C for 48-hr. Shell thickness was defined as the shell mass per area (mg mm-2) and condition index as tissue mass per unit shell mass (mg mg-2).

**Experimental conditions—** Oyster plates were exposed to target TA conditions at ambient or low salinity (S = 27) for 5 weeks, in isolated bins of circulating seawater. We targeted elevated, ambient and reduced TA conditions in ambient salinity (mean TA = 1000 =-, 1950 +-, 2900 +- µmol kg-1) and determined TA conditions in low salinity treatments as simulated mixes outcomes between ambient seawater (TA = 2250 µmol kg-1, S = 34) and distilled freshwater, a low TA freshwater source, or a high TA source (mean TA = 800 =-, 1500 +-, 2200 +- µmol kg-1). Oysters were fed daily and primarily kept in the dark (except for during water changes) to minimize the influence of shadows on activity (cite).

Experimental bins had lids but were not tightly sealed due to a gap created by the pump power cord, resulting in chemical drift between water changes. Although seawater temperature, salinity and TA exhibited variability over the course of the experiment, treatments remained independent (Figure 2). Continuous mixing with pumps ensured oxygen saturation remained high (> 80% cite), though, measurements from two bins did fall below 80% on two separate occasions due to pump motor failure (failed pumps were promptly replaced). Mortality of oysters was recorded at the same time as water changes, and dead shells were promptly discarded.

We performed complete seawater changes every third day to refresh conditions (see *Chemical manipulation of seawater* below), at which time bins and recirculating pumps were thoroughly cleaned. Prior to and again after water changes, we measured seawater temperature, salinity, pH voltage, and dissolved oxygen concentration with a handheld multi-parameter sonde (YSI X). Concurrently, we measured pH using spectrophotometric methods in a subset of the sonde measurements (calibrated daily with m-cresol dye standards, Easley and Byrne 2015), and used this relationship to convert voltage to total scale pH.

We collected and immediately froze 250ml seawater samples before and after water changes for later alkalinity determination. We quantified seawater total alkalinity concentration in triplicate using a Metrohm 855 Titrosampler, correcting titration acid concentration daily with certified reference materials from the laboratory of Dr. Andrew Dickson (Scripps Institute of Oceanography).

We then used measurements of seawater TA (µmol kg-1) and pH (total scale) at specified salinities and temperatures to estimate the remaining seawater carbonate system parameters with the *seacarb* package in R (version 3.3.1). In our *seacarb* estimates, we used equilibrium constants from Lueker et al. 2000 (K1 and K2), Perez and Fraga 1987 (Kf), and Dickson 1990 (Ks). For simplicity, we refer to the seawater carbonate system in terms of TA, though we emphasize that additional carbonate system parameters are required to fully describe seawater conditions (Table S1; see also Zeebe and Wolf-Gladrow 2001) and that these parameters influence oyster growth separate from TA (cite).

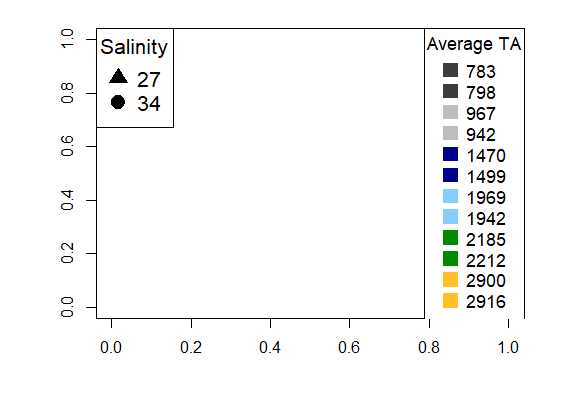
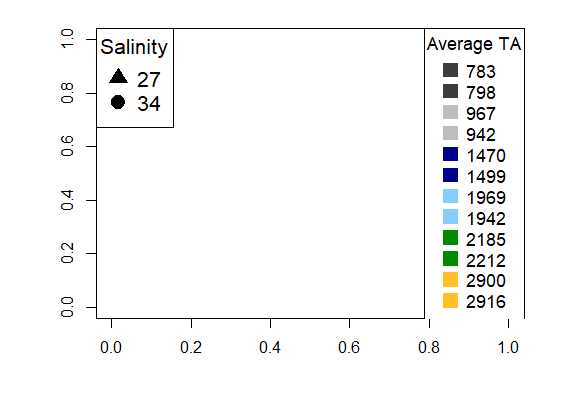
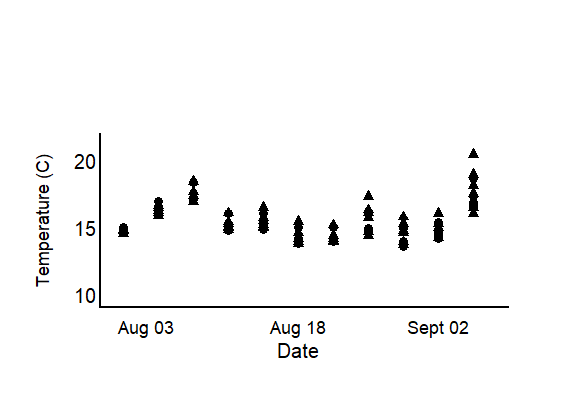
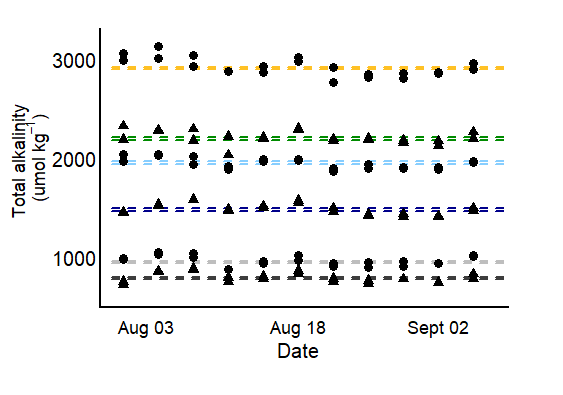


Fig. 2

**Chemical manipulation of seawater—**Every third day, we fully refreshed experimental seawater to prescribed TA conditions. First, we depleted seawater TA to negligible concentrations in large sumps (n = 4/ water change) by adding hydrochloric acid and strongly bubbling with ambient air, two days prior to each water change. This method drove the carbonate system equilibrium reactions towards CO2 and allowed it to off gas out of seawater. We then mixed this prepared seawater with distilled freshwater and adjusted the carbonate system to desired TA conditions with premade solutions of NaHCO3 (sodium bicarbonate) and Na2CO3 (sodium carbonate) with HCl (Waldbusser et al. 2015, Ninokawa et al. in review).

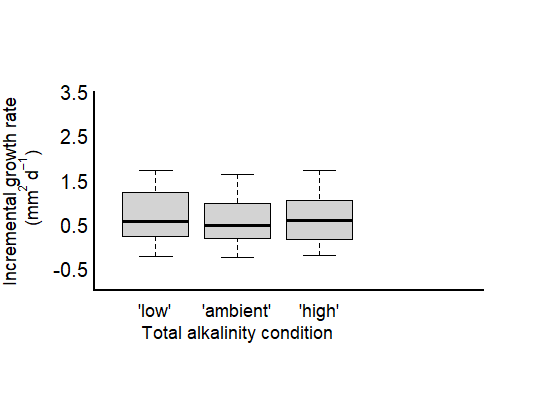
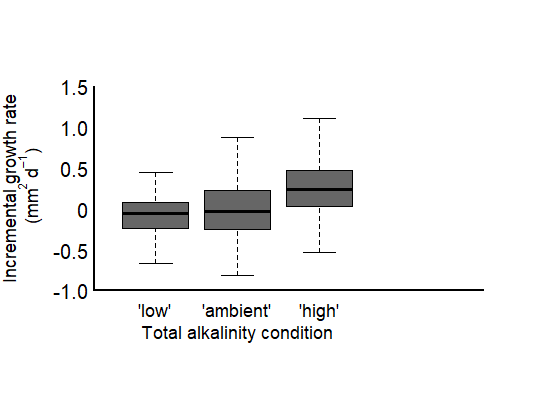
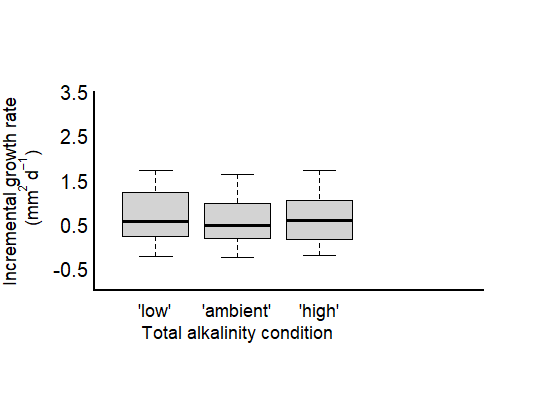
**Statistical analysis—** All statistical tests were performed in R Studio (ver. 2022.07.02). We used mixed effects models (*nlme*, *lme4*) to explore the how TA condition affects shell growth rate over the course of an exposure trajectory, relative to net responses exhibited. Due to logistical hurdles, we held multiple oysters in a single bin, which limited our degrees of freedom and prevented us from being able to test for the effect of salinity. Therefore, to maintain adequate power in our statistical analysis, responses to TA conditions will only be compared within similar salinities.

First, we wanted to understand whether initial oyster surface area growth rates (mm2 d-1) differed as a function of TA condition. For ambient and low salinity separately, we tested the influence of TA condition (as a factor) and initial surface area (continuous predictor) on initial growth as fixed predictors and included bucket as a random intercept to account for a lack of independence among oysters from the same plate. Then, we repeated this process for the latent growth period but included surface area at day 18 as a fixed predictor instead of initial size. We used similar models to test the influence of TA condition on overall surface area growth, including TA condition and initial surface area again as fixed predictors, separately for ambient and low salinity. The influence of TA condition on overall shell thickness (shell mass per area, mg mm-2) and condition index (tissue mass per shell mass, mg mg-2) was also tested, dropping the initial size term used in other models. In all models we included bucket as a random intercept. We added a weighted variance term to models that first failed the Breusch-Pagan test (*lmtest*) for residual heteroscedasticity, which specifies that the weight of each data point equal to the proportional variance across TA condition. Assumptions of normality were visually verified with qqplots and histograms of model residuals.

***Results—***

**Incremental surface area growth—** Surface area growth rates were higher in the initial period of the response trajectory, relative to later on, but did not initially differ among TA conditions within ambient nor lower salinity treatments (). The effect of TA condition on shell growth manifested in the later increment, where oysters exposed to the highest TA condition (within ambient or low salinity) tended to have elevated rates of surface area shell growth, though only in low salinity treatments was the trend found to be significant ().

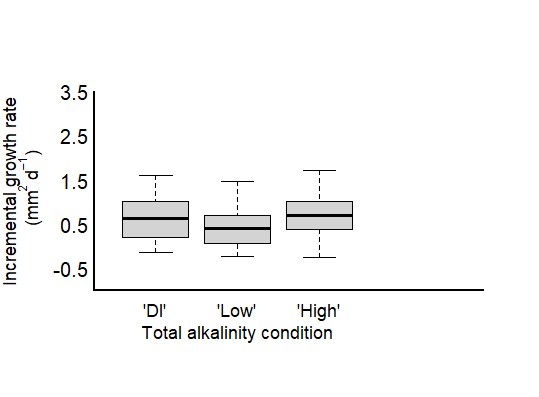
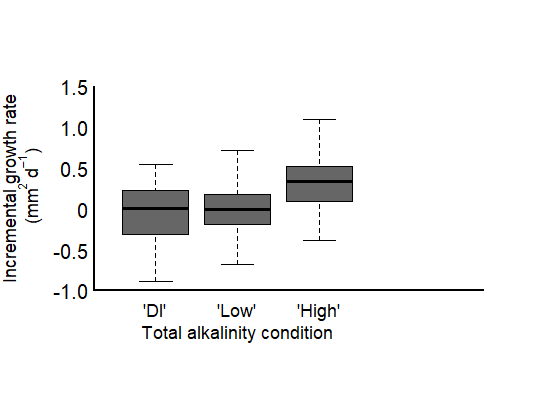
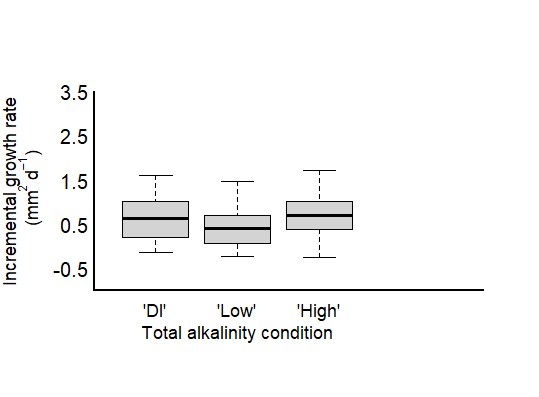
In addition to TA condition, size of the individual separately influenced oyster surface area growth. Oyster growth rates X as a function of initial shell area, indicating that surface area growth was X. We saw X effect of surface area on initial growth, and X effect in latent growth in ambient salinity. That is X to oysters in low salinity, where X happened.



*days 0 - 18*

*days 18 - 36*

Fig. 2



a

ab

b

*days 0 - 18*

*days 18 - 36*

Fig. 2

**Net growth—** The sum effect of TA condition and initial size on surface area growth was greater in oysters at lower salinity than those exposed to ambient. Specifically net surface area growth, like initial incremental growth, did not differ among TA conditions in oysters exposed to ambient salinity. Net surface area growth under lower salinity conditions was lower in DI TA conditions than high TA conditions, but low TA conditions did not significantly differ from either. Initial area size was again showing X.

Although TA impacted net lateral growth in some conditions, it did not influence average shell mass per area, a proxy for shell thickness, when coupled with either ambient or lower salinity. Similarly, we did not see a difference in oyster condition index across TA conditions or salinities. Average tissue mass greatly exceeded that of un-fed oysters held in lab seawater (X vs X) indicating an ability of all oysters to assimilate and store food as tissue mass. Condition index in high TA and lower salinity did not statistically differ, suggesting that oysters in this condition not only increased their surface area, but also tissue mass overall.

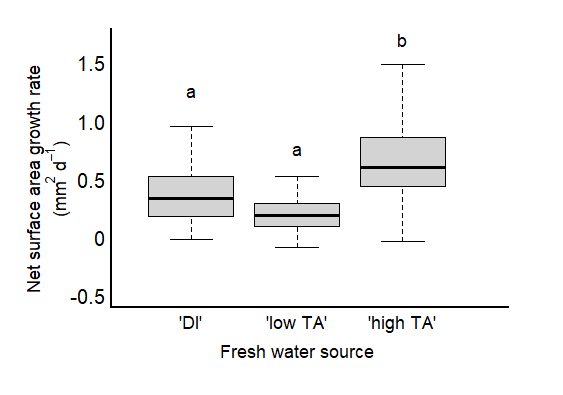
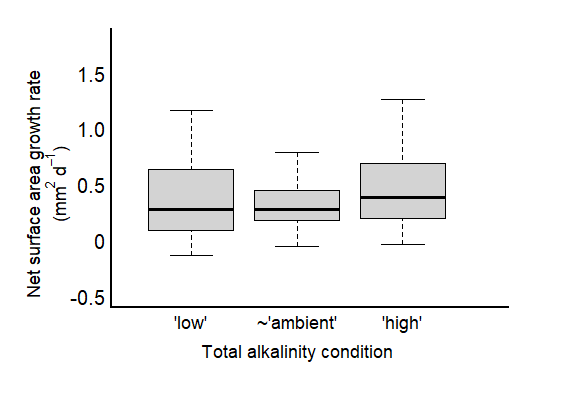
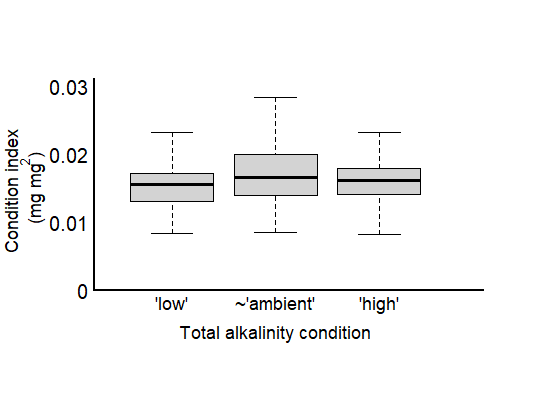
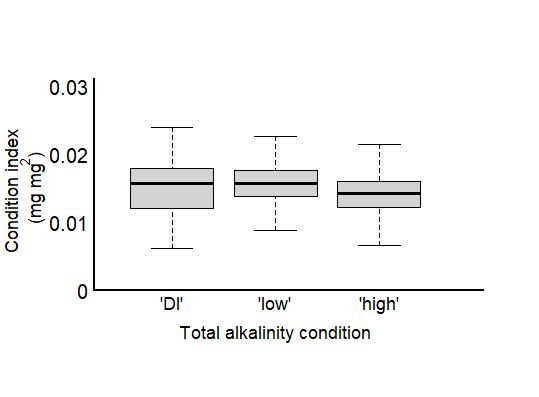
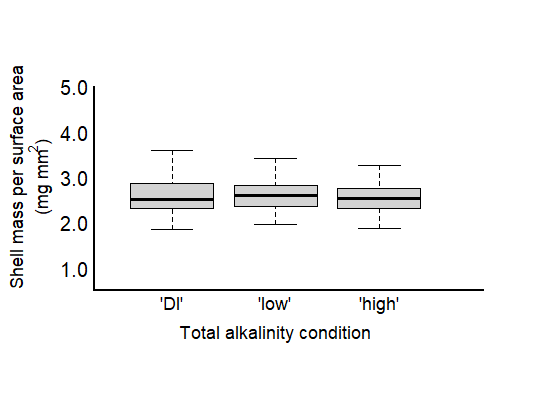
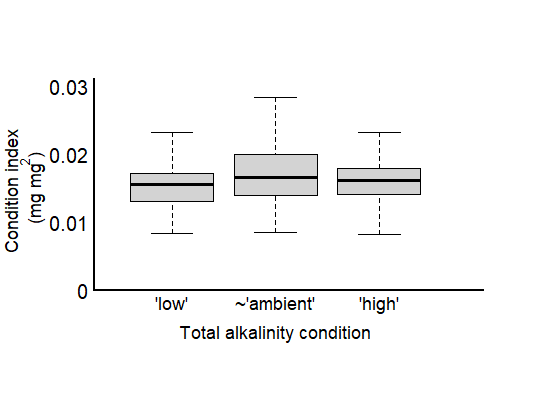
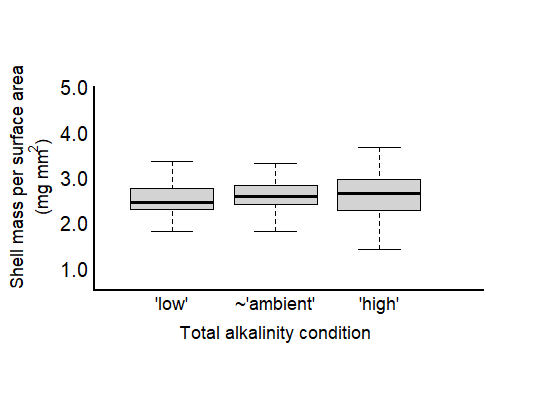


Fig. 4

A severe decline in growth rate between initial and latent increments could indicate a switch between shell growth and tissue growth, however, due to not wanting to sacrifice individuals following the first increment, we lack tissue mass samples to corroborate.



*(S = 34)*

*(S = 34)*

*(S = 27)*

*(S = 27)*

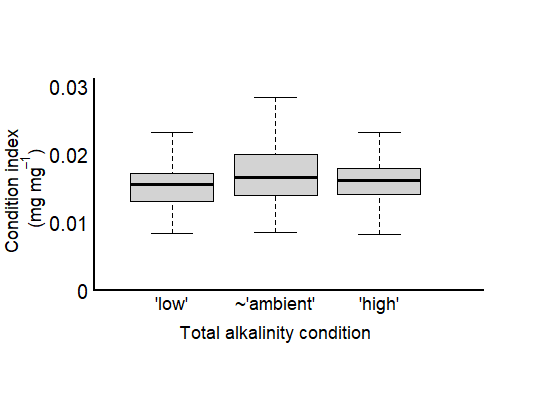
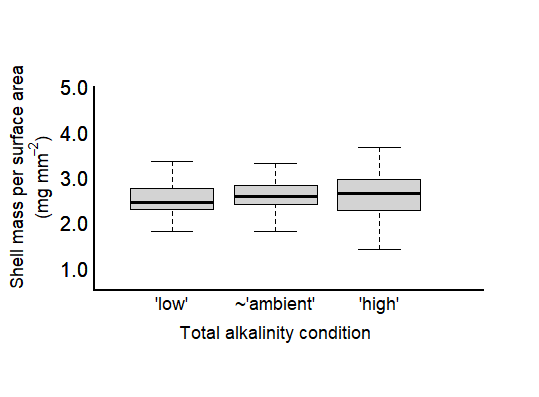


Fig. 2

***Discussion—***

Table X. Results of mixed effects, linear model testing the effects of factor(TA condition) and factor(experimental increment) on incremental surface area growth rates (mm2 d-1) in juvenile *Crassostrea virginica* oysters grown in **ambient salinity**. Change in surface area is relative to the size of the oyster at the beginning of the experimental increment (initial (0-18) or at day 18 (18-36). L-Ratios and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. The final model: Incremental growth rate ~ factor(TA condition) + factor(experimental increment) + RI(bin) + weight = by(TA condition), accounted for ~ X% of the variation.

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| --- | --- | --- | --- | --- | --- | --- |
| **Model A: Initial (0-18 days) surface area growth rate (mm2 d-1) in ambient salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.62  cond. r2 = 0.63 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
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| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |

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| **Model B: Latent (18 - 36 days) surface area growth rate (mm2 d-1) in ambient salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.31  cond. r2 = 0.35 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
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| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |

Table X. Results of mixed effects, linear model testing the effects of factor(TA condition) and factor(experimental increment) on incremental surface area growth rates (mm2 d-1) in juvenile *Crassostrea virginica* oysters grown in **low salinity**. Change in surface area is relative to the size of the oyster at the beginning of the experimental increment (initial (0-18) or at day 18 (18-36). L-Ratios and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. The final model: Incremental growth rate ~ factor(TA condition) + factor(experimental increment) + RI(bin) + weight = by(TA condition), accounted for ~ X% of the variation.

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| --- | --- | --- | --- | --- | --- | --- |
| **Model A: Initial (0-18 days) surface area growth rate (mm2 d-1) in low salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.62  cond. r2 = 0.63 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
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| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
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| **Model B: Latent (18 - 36 days) surface area growth rate (mm2 d-1) in low salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.31  cond. r2 = 0.35 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
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| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |

Table X. Results of mixed effects, linear model testing the effects of factor(TA condition) and factor(experimental increment) on surface area growth rates (mm2 d-1) in juvenile *Crassostrea virginica* oysters grown in **ambient or low salinity**. Change in surface area is relative to the size of the oyster at the beginning of the experimental increment (initial (0-18) or at day 18 (18-36). L-Ratios and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. The final model: Incremental growth rate ~ factor(TA condition) + factor(experimental increment) + RI(bin) + weight = by(TA condition), accounted for ~ X% of the variation.

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| **Model A: Net surface area growth rate (mm2 d-1) in ambient salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.62  cond. r2 = 0.63 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
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| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |
| **Model A: Net surface area growth rate (mm2 d-1) in low salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.62  cond. r2 = 0.63 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
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| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |

Table X. Results of mixed effects, linear model testing the effects of starting size (mm2) and TA condition (factor) on (a) net surface area growth rates (mm2 d-1) and (b) shell mass per area in juvenile *Crassostrea virginica* oysters grown in **ambient** salinity. L-Ratios and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. The final models, Net growth rate ~ starting size + factor(TA condition) + RI(bin) + weight = by(TA condition) and Shell mass per area ~ starting size + factor(TA condition) + RI(bin) + weight = by(TA condition) accounted for ~ X and X% of the variation, respectively.

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| **Model A: Net growth, Shell mass per shell area (mg mm-2) in ambient salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.24  cond. r2 = 0.41 | **Estimate** | **Std. Error** | **t-value** | **df** | **L. Ratio** | **p-value** |
| Intercept (factor (low TA)) |  |  |  |  |  |  |
| Starting size (mm2) |  |  |  |  |  |  |
| factor (mid TA) |  |  |  |  |  |  |
| factor (elevated TA) |  |  |  |  |  |  |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin | **z** |  |  |  |  |  |

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| **Model B: Net growth, Condition index in ambient salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.02  cond. r2 = 0.02 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
| Intercept (factor (low TA)) |  |  |  |  |  |  |
| factor (mid TA) |  |  |  |  |  |  |
| factor (elevated TA) |  |  |  |  |  |  |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |

Table X. Results of mixed effects, linear model testing the effects of starting size (mm2) and TA condition (factor) on (a) net surface area growth rates (mm2 d-1) and (b) shell mass per area in juvenile *Crassostrea virginica* oysters grown in **low** salinity. L-Ratios and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. The final model: The final models, Net growth rate ~ starting size + factor(TA condition) + RI(bin) + weight = by(TA condition) and Shell mass per area ~ starting size + factor(TA condition) + RI(bin) + weight = by(TA condition) accounted for ~ X and X% of the variation, respectively. We tested for differences in the TA conditions by looking for overlap in confidence intervals given by the model and are labeled as such.

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| --- | --- | --- | --- | --- | --- | --- |
| **Model A: Net growth, shell mass per shell area (mg mm-2) in low salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.45  cond. r2 = 0.54 | **Estimate** | **Std. Error** | **t-value** | **df** | **L. Ratio** | **p-value** |
| Intercept (factor (low TA)) |  |  |  |  |  |  |
| Starting size (mm2) |  |  |  |  |  |  |
| factor (mid TA) |  |  |  |  |  |  |
| factor (elevated TA) |  |  |  |  |  |  |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Model B: Net growth, Condition index in low salinity** | | | | | | |
| **Fixed Effects**  marg. r2 = 0.02  cond. r2 = 0.02 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
| Intercept (factor (low TA)) |  |  |  |  |  |  |
| factor (mid TA) |  |  |  |  |  |  |
| factor (elevated TA) |  |  |  |  |  |  |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Bin |  |  |  |  |  |  |