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Synergistic effects of salinity and temperature on the eastern oyster *Crassostrea virginica* throughout the lifespan

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ABSTRACT: The eastern oyster *Crassostrea virginica* has a wide salinity tolerance, but all life stages are vulnerable to environmental extremes, and elevated temperatures can truncate the expected salinity tolerance. The rising water temperatures and more intense and variable storm events predicted to accompany global climate change therefore raise concerns for habitat suitability for ecologically important species such as the eastern oyster. To better understand environmental limitations, oysters of all life stages were exposed to a range of salinities (0–40) and temperatures (25 and 30°C) to test physiological tolerance to the combined effects of osmotic and thermal stresses. Elevated temperatures (30°C) amplified negative effects during exposure to salinity extremes at all life stages; however, tolerance to extremes increased with developmental stage (gametes < embryos < larvae < spat < adult). Gradual changes allow for a wider tolerance in juvenile oysters (spat) compared to acute changes, and short-term reprieves during low salinity exposure improved survival rates for adults. Overall, the present study found a threshold salinity of 15 for polyhaline oyster populations and highlights the importance of both rate of change and temperature as critical components of salinity tolerance. Additionally, salinities <10 during the summer months could result in negative population effects, especially if extreme low salinity occurs during peak reproduction when pelagic gametes, embryos, and larvae are most vulnerable to environmental stresses. This work will benefit population models and inform resource management decisions regarding the timing of controlled freshwater releases.

KEY WORDS: Gametes · Embryos · Larval invertebrates · Juveniles · Growth · Osmotic stress · Thermal stress · Oysters

1. INTRODUCTION

Estuaries are highly productive ecotones that provide essential ecosystem services such as coastal resilience, species diversity, and significant economic value to coastal communities. Serving as a permeant home, spawning grounds, nursery habitat, and/or foraging grounds for numerous species, it is estimated that 68 and 80% of US commercial and recreational landings, respectively, are estuarine dependent (Lellis-Dibble et al. 2008). Estuaries also buffer between land and sea to protect against storm events and prevent shoreline erosion, but their ability to function as a buffer is dependent on the living organ-

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isms that comprise the estuary to maintain its structure and integrity. Oysters are one of the most critical ecosystem engineers in these systems. After a short (~2 wk) pelagic larval distribution phase, oyster larvae settle out of the water column to form permanent, complex, 3-dimensional reef structures that both serve as a refuge for small estuarine species and natural breakwaters to protect from erosion and aid in sedimentation (Piazza et al. 2005, Grabowski & Peterson 2007). As filter feeders, oysters also contribute to the estuary's role in serving as a natural filtration system between the land (rivers and tributaries) and the open ocean. In addition to trapping sediments, their natural filter feeding behavior removes phytoplankton and excess nutrients from the water column, abating the effects of eutrophication and improving water clarity (Newell 2004, Grabowski & Peterson 2007, Grizzle et al. 2008).

Estuaries are dynamic with daily and seasonal changes in salinity as a characteristic-defining factor that drives species distribution (Sklar & Browder 1998, Elliott & McLusky 2002). The eastern oyster *Crassostrea virginica* (Gmelin, 1971) possesses incredible phenotypic plasticity to cope with this dynamic environment, tolerating salinities as low as 2–3 (Southworth et al. 2017, McCarty et al. 2020, 2022) and as high as 40 (Galtsoff 1964) for prolonged periods of time when the change is gradual (e.g. seasonal variation in rainfall) (McFarland et al. 2022), and for short periods of time they can accommodate rapid salinity changes by secluding themselves from the external environment through shell closure (Loosanoff 1952, Shumway 1977). The response to salinity is well studied for the eastern oyster, but in the abundance of research, it is clear that specificity of response and limitations vary not only across life stages but also among populations, largely dependent on previous exposure history. A salinity range of 14–28 is often highlighted as optimal (Shumway 1996); however, low salinity regions (<12) can provide refuge from disease and predation and have been shown to support thriving oyster reefs (La Peyre et al. 2003, 2016, Ford et al. 2012). In the upper Chesapeake Bay, Maryland, USA, an optimal range for larvae produced from mesohaline broodstock (salinity of 10–11.2) was 7–16 (Scharping et al. 2019) and in the Hudson River, New York, USA, active gametogenesis and recruitment are observed in the upper estuary at salinities <5 and <10, respectively (McFarland & Hare 2018). In contrast, oyster reefs experiencing moderately high salinities (15 yr average of 19) are resilient to extreme low salinity events (<5) in the winter months when temperature is low (<20°C), but extensive mortality events ensue when low salinity events (<10) occur during the summer when temperatures are elevated and gametogenesis is at a peak (La Peyre et al. 2013, McFarland et al. 2022). Salinity tolerance is a heritable trait (McCarty et al. 2020, 2022) and is likely to play an important role in differences observed among populations. In Delaware Bay, Delaware, USA, larvae produced from low (6.5–14.5) and high (20–25) salinity broodstock populations performed better in their home salinity even after broodstock had 14 wk conditioning in the test salinity (10 or 30) (Eierman & Hare 2013). From these same populations, oysters collected from the high and low salinity reefs also showed a significant genetic by environment interaction in a reciprocal mesocosm exposure to low and high salinity (10 and 30) conditions (Eierman & Hare 2016). This highlights the need for understanding environmental tolerance at the population level that includes an understanding of previous exposure history.

Elucidating the combined effects of multiple stressors is essential to better understand and model resiliency under dynamic conditions experienced in the natural setting. Thermal stress alone can impede growth and development in bivalve larvae (Loosanoff 1965), and the effect is amplified when accompanied by other environmental stressors, often exerting a synergistic negative effect on the organism (Przeslawski et al. 2015). Elevated temperatures can further limit osmotic tolerance of oysters due to increased metabolism leaving less energy to be allocated towards homeostasis and cellular repair, particularly when temperature falls outside of the preferred range (Berger & Kharazova 1997, Sokolova et al. 2012, Rivera-Ingraham & Lingot 2017, Casas et al. 2018a). Both field and laboratory studies highlight the critical interaction between salinity and temperature on the survival of oysters and the differential response (growth and survival) among different size/age classes to the combined stressors (Rybovich et al. 2016, Southworth et al. 2017, McFarland et al. 2022). Rybovich et al. (2016) found market-sized oysters (>75 mm) to be most sensitive to low salinity exposures (1, 5, 15) followed by seed oysters (25–75 mm) and the best survival in spat (<25 mm). For all size classes, mortality was significantly greater when temperature was also elevated (32°C). The free-swimming pelagic life stages (embryos and larvae) are particularly vulnerable, requiring a narrower salinity and temperature range than observed for post-metamorphic oysters (spat, seed, adults) (Shumway 1996). Alone, temperature limits the duration of gamete viability (Carriker 1996), and embryogenesis is limited at low salinities

(Davis & Calabrese 1964). The synergistic effects of thermal and osmotic stress not only limit embryogenesis and larval development but also increase susceptibility to other environmental stressors (MacInnes & Calabrese 1979, Ko et al. 2014).

The already observable effects of climate change demand the need to take a multistressor approach to organismal response to change and highlight temperature as an imminent concern for marine organisms. Globally, 2021 set the record for the hottest ocean temperatures and was preceded by record breaking years in 2020 and 2019 (Cheng et al. 2022). Warming oceans result in increased precipitation that result in increased freshwater input to coastal systems and depressed salinity (IPCC 2013). Thus, warming water temperatures, coupled with more variable and intense storm and rainfall events, can result in rapid changes to the salinity gradient and may be particularly detrimental in coastal regions that have experienced extensive anthropogenic alterations to the morphology of coastlines and drainage patterns (Sklar & Browder 1998, Scavia et al. 2002). For many regions, extreme low salinity events are common in the summer months when temperatures are elevated and reproductive activity is at its peak, leaving early life stages vulnerable to extreme changes in environmental conditions. Many coastal regions have also undergone dramatic modifications to the natural water flow and drainage patterns which can intensify flood or drought periods (e.g. McFarland et al. 2022). Controlled freshwater releases or intentional withholding of freshwater into estuaries can cause rapid changes to the salinity gradient within an estuary on a timescale of days to hours (Volety et al. 2009, McFarland et al. 2022). The timing of these events is therefore crucial to long-term population success and must be considered in restoration planning and management decisions regarding land usage and controlled freshwater releases. Interrupting larval production and/or recruitment can have lasting effects at the population level (Underwood & Fairweather 1989), and understanding the environmental boundaries of the pelagic larval stage can help to predict the response of local population structure and dynamics. Evaluating response variables to the synergistic effects of multiple drivers across all life stages within a single population will also improve the ability to model changes in tolerance over the lifespan as development, age, and physiological status all contribute to sensitivity to environmental stress.

Here we evaluate the osmotic limits of the eastern oyster under moderate and elevated temperatures across all life stages in a polyhaline population. These data are critical for modeling efforts to project population level response to future salinity and temperature regimes under climate change scenarios. By testing tolerance at several critical developmental stages, fertilization, embryogenesis, D-hinge larvae, juveniles (i.e. spat), and adults within a population, these data can better inform model parameters that require full life stage input.

2. MATERIALS AND METHODS

2.1. Collection and maintenance of organisms

Adult oysters were collected during the natural spawning season (March–July 2014) from the Caloosahatchee River Estuary, Ft. Myers, Florida, USA, where average annual salinities remain moderately high (~19), but seasonal drought and flood events often result in extreme hypersaline and hyposaline exposures, subsequently leading to mortality events (McFarland et al. 2022). All oysters were held in conditioning tanks for approximately 4–6 wk prior to spawning in the laboratory. Broodstock were held under chilled water conditions (~20°C) to prevent premature spawning, under ambient salinity (~25) and fed a mixture of Shellfish Diet 1800® (Reed Mariculture) and live phytoplankton (*Chaetoceros* sp., *Tetraselmis chui*, and *Tisochrysis lutea*) at a daily ration of 3% dry mass (Utting & Millican 1997). Oysters were spawned using thermal cycling procedures at a salinity of 25 by slowly increasing the temperature from 20 to 28°C. If the exposure to the warm water treatment did not elicit spawning within 30 min, the oysters were transferred back into the cold water tank (16°C) for 30 min. This process was repeated until spawning occurred. As spawning commenced, individuals were removed from the spawning table and allowed to finish releasing gametes in separate beakers for the collection and isolation of gametes. After a subset of pooled gametes were removed for challenge experiments (see section 2.2.1), eggs were fertilized at a ratio of approximately 100 sperm per egg. One hour after fertilization, a subset of embryos was removed for challenge experiments (see section 2.2.2), and the remaining embryos were transferred to larval incubation tanks for hatching. Following hatch, 2 d old larvae (D-stage) were used for larval challenge experiments. Individual (cultchless) juvenile oysters (spat; 10–15 mm) were obtained from Bay Shellfish Company, and adult oysters were collected from natural reefs in the Caloosahatchee

River Estuary. The inability to collect enough juveniles from the same reefs resulted in the need to use hatchery produced oysters from Bay Shellfish. Bay Shellfish broodstock were wild collected oysters, not selectively bred lines for aquaculture, and from a similar environment to those collected from the Caloosahatchee River Estuary in regard to their previous temperature and salinity exposure. All experimental oysters (spat and adults) were held for a minimum of 2 wk at a salinity of 25 and ambient naturally fluctuating temperature prior to experimentation to allow for acclimation to laboratory conditions and were fed a combination of Shellfish Diet 1800® and live phytoplankton.

2.2. Exposure to gametes and early life stages

Gamete, embryo, and larval exposures were conducted in 400 ml beakers with gentle aeration and held inside an incubator to maintain controlled temperatures (see subsections for each life stage below). Oysters from each life stage were exposed to salinities of 0, 3, 5, 10, 15, 20, 25, 30, 35 and 40 at 2 temperature treatments, 25 and 30°C (4 replicates per treatment). The 2 temperatures reflect temperatures observed in this region during spring (25°C) and summer (30°C) spawning periods (McFarland et al. 2022) providing an estimate of current effects of extreme salinity exposure. The desired salinity was achieved by mixing filtered seawater with either Instant Ocean® or deionized water to achieve treatment salinity. All exposures were acute changes from the control treatment at a salinity of 25 at 25°C. During experiments, larvae were fed a mixture of live algae (*Chaetoceros* sp. and *T. lutea*) to reach a background concentration of approximately 10 000 cells ml^{-1}, and environmental conditions (temperature and salinity) within experimental containers were monitored daily. At each sampling time, larvae were mixed well within the beaker, and a 10 ml sample was removed and fixed with 10% buffered formalin. Fixed samples were subsampled 3 times by withdrawing 300 µl, and larvae were examined using a Wildco® Sedgewick-Rafter Counting Cell Slide under a microscope for volumetric counts and photomicrographs. Fertilization success, hatch rate (or D-larval yield), mortality, developmental abnormalities, and shell lengths (n = 25 per replicate) were quantified when applicable for all treatments and exposures. Abnormal development was identified by assessing the shape of the shell and velum, which could affect the ability to fully close shells, and/or feeding and respiratory function, as well as by ennumerating arrested development such as embryos or trocophores at later time points (His et al. 1999). See Fig. S1 in the Supplement (www.int-res.com/articles/suppl/m700p111_supp.pdf) for experimental work flow of gamete and early life stage exposures.

2.2.1. Gamete exposure

Gamete exposure was carried out by first exposing eggs and sperm to the various salinity and temperature treatments separately prior to fertilization. Eggs were added to the experimental chamber first (approximately 4000 eggs $replicate^{-1}$; 10 eggs ml^{-1}), and sperm was exposed simultaneously in a 50 ml beaker at the corresponding salinity and temperature. After 30 min of exposure, 10 ml sperm was added to the eggs to obtain a ratio of approximately 100:1 (sperm:egg). Fertilization rate, hatch rate, mortality, developmental abnormalities, and growth were quantified by sampling after 1, 24, and 96 h and analyzed microscopically. Fertilization rates were calculated as the ratio of embryos showing cell division to unfertilized eggs. Hatch rates were calculated as a ratio of D-stage larvae to undeveloped embryos after 24 h. Shell length was measured for a minimum of 25 larvae per replicate.

2.2.2. Embryo exposure

Fertilized embryos, from gametes fertilized at a salinity of 25 and 25°C, were used to assess embryogenesis and larval development under the various treatment conditions. Embryos were exposed 1 h after fertilization by transferring approximately 4000 embryos (10 embryos ml^{-1}) to experimental chambers at each salinity and temperature treatment. Larvae were sampled at 48 and 96 h, and hatch rate, mortality, shell length, and developmental abnormalities were quantified microscopically. Shell length was measured for a minimum of 25 larvae per replicate.

2.2.3. Larval exposure

D-stage larvae (48 h post fertilization), previously fertilized and hatched at a salinity of 25 and 25°C, were used for larval exposures to the various treatment conditions. Approximately 3000 larvae (8.5 larvae ml^{-1}) were transferred into beakers at each of the

experimental salinities and sampled after 96 h of exposure to experimental conditions. Development, survival, and growth were examined microscopically. Shell length was measured for a minimum of 25 larvae per replicate.

2.3. Juvenile exposure to acute and gradual salinity changes

One month old juvenile oysters (10–15 mm) were exposed to a range of salinity (0, 3, 5, 10, 15, 20, 25, 30, 35 and 40) and temperature (25 and 30°C) treatments using 600 ml beakers (n = 20 spat beaker^{-1}; 3 replicates treatment^{-1}). Final treatment salinity was achieved by mixing filtered seawater with either Instant Ocean® or deionized water. Oysters were fed daily by adding 0.5 ml Shellfish Diet 1800® per beaker, and water was changed every other day at which time oysters were carefully inspected for mortality. An oyster was considered dead if it was gaping and did not close when prodded. Exposures consisted of both acute and gradual salinity changes, but temperature change was acute only. During acute salinity changes, oysters were taken directly from holding conditions at the control salinity (25) and placed into the experimental treatment salinity where conditions were maintained for 14 d. During gradual salinity exposures, all treatments started at a salinity of 25 and their respective temperature. Water changes were completed every other day at which time salinity was increased or decreased by 2–3 until the desired salinity was achieved. A control salinity was maintained at 25 for both temperatures, and water changes continued after salinity was reached to maintain water quality. All treatment salinities were reached by Day 20 of the adjustment period, following which the experimental treatment conditions were maintained for 10 additional days to assess survival at the target salinity, resulting in a total experimental duration of 30 d.

2.4. Adult salinity exposure

Adult oysters (mean shell length = 64.42 ± 1.4 mm) were exposed to fluctuating salinities to simulate the effect of pulsed freshwater releases (cycles) and prolonged freshwater releases (continuous), compared to controls maintained at a salinity of 25. Low salinity treatments were determined based on local seasonal regimes (McFarland et al. 2022) and consisted of 4 regimes: salinity of 3 continuous, 7 continuous, 3 for 10 d with a 5 d increase to 10 then back to 3 (3/10 cycle), and 7 for 10 d with a 5 d increase to 14 then back to 7 (7/14 cycle). These exposures aimed to simulate a wetter than average wet season (3 and 3/10) compared to a moderate wet season (7 and 7/14), based on local data (McFarland et al. 2022). Oysters were exposed by gradually decreasing the salinity (~3 d^{-1}) from holding conditions (25) to treatment salinities over a 1 wk period. Experiments were conducted in aerated 40 l aquaria with 25 oysters per tank and 5 replicate tanks per treatment. Temperature was maintained at 25°C, and oysters were fed Shellfish Diet 1800® at a rate of approximately 3% dry biomass d^{-1}. Oyster survival was monitored daily, and full water changes were conducted every 3 d to maintain optimal water quality. At the start of the exposure period (t = 0), 20 oysters were destructively sampled for baseline condition index measurements. At the end of the exposure (Day 25), all remaining live oysters were destructively sampled for measurement of condition index. Condition index was measured according to Lucas & Beninger (1985). Briefly, whole oyster tissue was dissected out of the shell and both tissue and shell were dried at 60°C for 48 h and weighed. Condition index was calculated as ([weight of dry tissue (g) / weight of dry shell (g)] × 100). Higher condition index values indicate better physiological condition.

2.5. Statistical analysis

Response variables (fertilization rate, hatch rate, mortality, developmental abnormalities, shell length) of early life stages and juvenile oysters exposed to variable salinity and temperature were tested using 2-way analysis of variance (ANOVA) to detect differences between salinity, temperature, and the interaction between the two. When a significant difference was detected, a post hoc Tukey's HSD was used to detect differences among salinity treatments. Kaplan Meier survival analysis was completed for the juvenile oyster exposures to assess mean survival time among all treatments. Adult exposure to low salinity cycles was examined using a 1-way ANOVA to test responses of survival and condition index to salinity treatment, followed by a post hoc Tukey's HSD to detect differences among treatments. Percent data were arcsine square-root transformed to meet assumptions of normality and heteroscedasticity. Statistical analysis was performed in SPSS 24. Results are represented as means ± SE to a significance level of $p < 0.05$.

3. RESULTS

3.1. Laboratory exposures of adult, juvenile, and early life stages to depressed salinity

3.1.1. Early life stages: gametes

When gametes were exposed to treatment conditions prior to fertilization, fertilization success was significantly affected by the interaction of salinity and temperature ($F_{9, 60}$ = 3.88; p < 0.001 (Table S1)) with complete inhibition of fertilization at salinities ≤10 at 30°C, and only 2% normal fertilization at salinity of 40 and 30°C (Fig. 1A). Fertilization was more successful at 25°C with 19 ± 7% and 35 ± 4% (mean ± SE) normal fertilization at salinities of 10 and 40, respectively; however, it was markedly inhibited at salinities <10 (Fig. 1A). Gamete exposure to salinites of 15–35 resulted in high fertilization rates (≥75%) for both temperature treaments (Fig. 1A). Although fertilization success was low at salinities 10 and 40 for 25°C, hatch rates of those with successful fertilization were relatively high (100 ± 0 and 84 ± 4.3%, respectively) compared to ≥89% in salinity treatments of 15–35 (Fig. 1B). Similar to fertilization rate, there was a significant interaction of salinity and temperature ($F_{6, 51}$ = 15.042; p < 0.001) on hatch rate. Among those treatments with successful fertilization, the lowest hatch rates were observed in the high temperature and salinity treatment (40 and 30°C), but only one replicate had normal fertilization, and 77% mortality was observed after 96 h (Fig. 1D). For treatments that had successful fertilization and hatch, both temperature ($F_{1, 33}$ = 14.556; p < 0.001) and salinity ($F_{5, 33}$ = 4.78; p = 0.002) had significant effects on larval survival after 96 h. For both temperature treatments, ≤10% mortality was observed at salinities 15–35 (Fig. 1D). The proportion of abnormal development after 96 h was

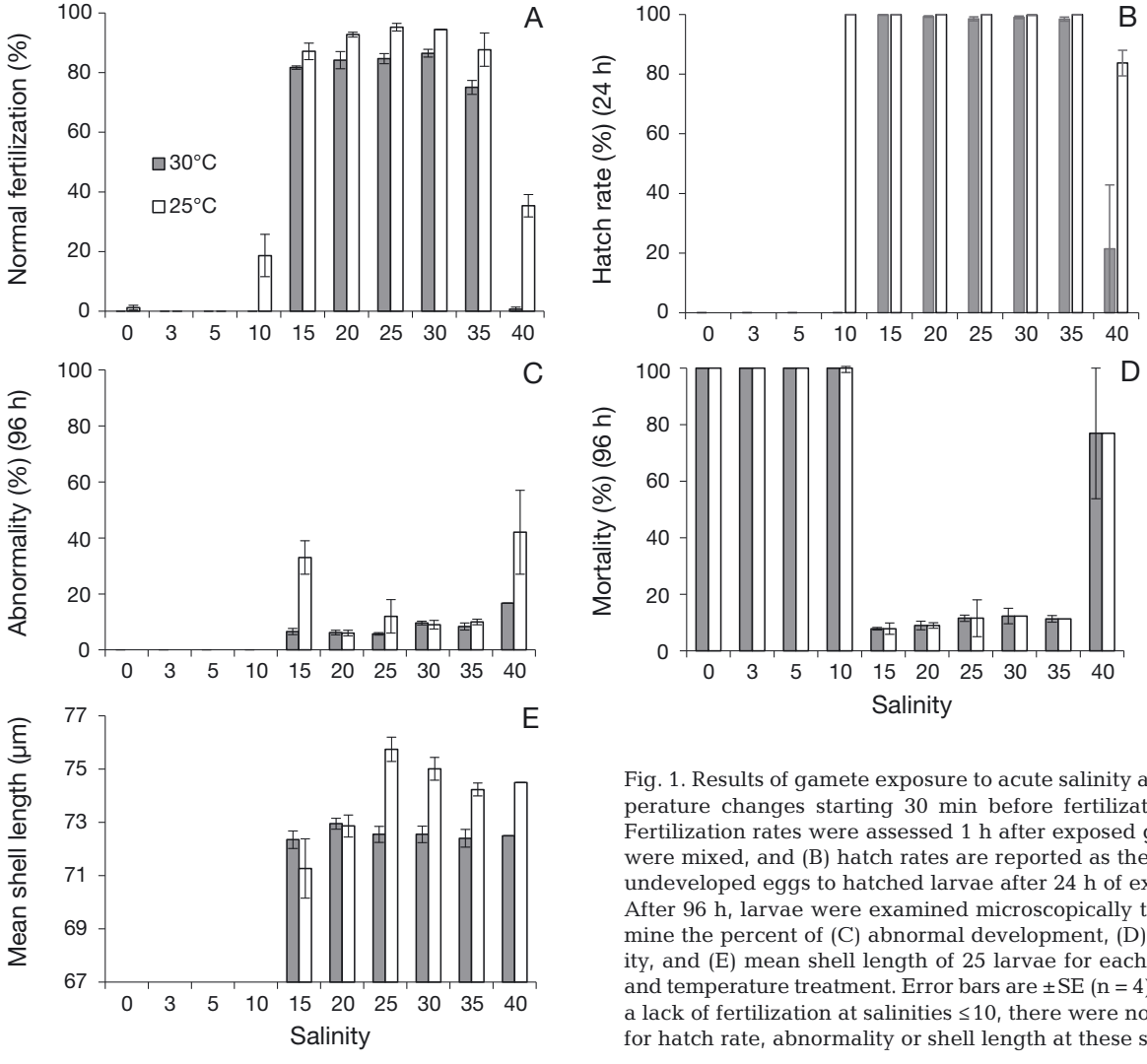

Fig. 1. Results of gamete exposure to acute salinity and temperature changes starting 30 min before fertilization. (A) Fertilization rates were assessed 1 h after exposed gametes were mixed, and (B) hatch rates are reported as the ratio of undeveloped eggs to hatched larvae after 24 h of exposure. After 96 h, larvae were examined microscopically to determine the percent of (C) abnormal development, (D) mortality, and (E) mean shell length of 25 larvae for each salinity and temperature treatment. Error bars are ±SE (n = 4). Due to a lack of fertilization at salinities ≤10, there were no reports for hatch rate, abnormality or shell length at these salinities

significantly affected by the interaction of salinity and temperature ($F_{5, 33}$ = 119.842; p < 0.001). Salinity treatments that experienced high survival (15–35) had relatively low incidence of abnormality (<12%), except 15 at 25°C for which 33% abnormal development was observed (Fig. 1C). Larval growth was significantly affected by salinity ($F_{6, 32}$ = 6.653; p < 0.001) and temperature ($F_{1, 32}$ = 43.544; p < 0.001) (Table S1), but no significant difference between temperature treatments was detected at salinities of 15–20. However, shell length was greater in the treatments held at 25°C for salinities of 25–40 (Fig. 1E). Although high growth was reported for salinity 40 at 30°C, this included only one replicate due to high mortality (100% mortality in 3 out of 4 treatments) and high mortality in the remaining replicate (77%) and, therefore, should be interpreted with caution.

3.1.2. Early life stages: embryos

Hatch rate of embryos was significantly affected by a salinity and temperature interaction ($F_{8, 57}$ = 82.46; p ≤ 0.001) (Table S2). No viable larvae were observed to have hatched after 24 h of exposure at salinities ≤5 at 25°C and salinities ≤10 or at a salinity of 40 at 30°C (Fig. 2A). After 96 h of exposure, mortality was significantly affected by salinity ($F_{8, 39}$ = 33.953; p ≤ 0.001), but no differences were observed between temperature treatments. For those larvae that did hatch at salinities 10 and 40 in the 25°C treatment, 100% mortality was observed after 96 h (Fig. 2B). No significant difference was observed in mortality between temperature treatments at salinities of 15–25. Abnormalities were relatively low in treatments that had successful hatch and survival to 96 h (Fig. 2C) but were significantly affected by the interaction between salinity and temperature ($F_{4, 31}$ = 11.01; p ≤ 0.001) (Table S2). Higher abnormality was observed in the 25°C treatments at salinities 15 and 35 compared to 30°C but remained low for both temperature treatments at salinities 20–30. Overall, larval growth was significantly affected by both salinity ($F_{5, 31}$ = 7.349; p < 0.001) and temperature ($F_{1, 31}$ = 263.43; p ≤ 0.001). Shell length after 96 h was higher at salinities of 20–30 for the 25°C treatment, but at a salinity of 35 growth was higher in the 30°C treatment (Fig. 2C).

3.1.3. Early life stages: larvae

Exposure of D-stage larvae to acute salinity and temperature changes resulted in a significant tem-

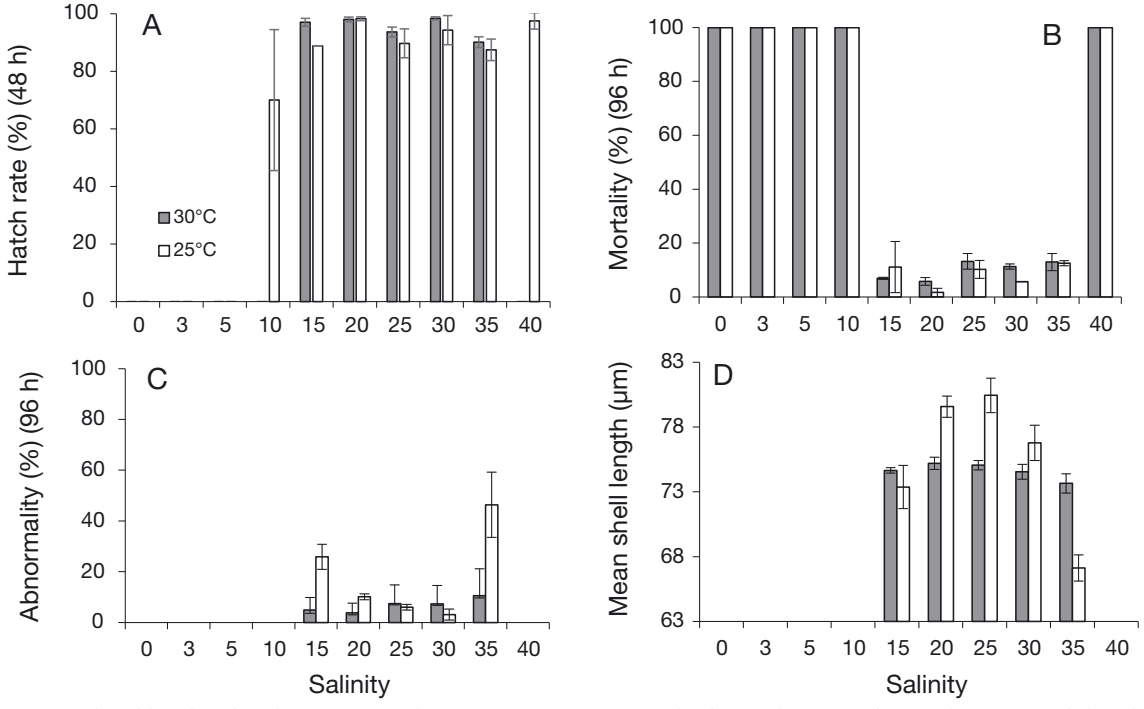

Fig. 2. Results of fertilized embryos exposed to acute temperature and salinity changes. (A) Hatch rates are defined by survival after 48 h of exposure. Larvae were assessed after 96 h for quantification of (B) mortality, (C) abnormality, and (D) mean shell length of 25 larvae among treatments. Abnormality and shell lengths are not reported for salinities ≤10 due to 100% mortality in these treatments. Error bars are ±SE (n = 4)

perature and salinity interaction affecting both mortality ($F_{9, 60}$ = 2.351; p = 0.024) and abnormality rates ($F_{8, 54}$ = 3.327; p = 0.004) of developing larvae (Table S3). Low salinity treatments of 0 and 3 resulted in 100 and 30–50% mortality, respectively (Fig. 3A), and for both temperature treatments abnormality of surviving larvae at a salinity of 3 was high (> 80%) (Fig. 3B). Abnormal development at a salinity of 3 increased in the high temperature treatment condition (Fig. 3B). At all other salinities (5–40), mortality and abnormality rates were low (<20%) for both temperature treatments. Growth was significantly affected by an interaction between salinity and temperature ($F_{8, 52}$ = 17.82; p < 0.001). Larger shell lengths were obtained at 25°C, with final lengths exceeding 80 µm in all salinity treaments ≥10, while at 30°C final length only reached 75 µm at salinities of 15 and 20 (Fig. 3C).

3.2. Juvenile oyster exposure

During acute salinity challenges to juvenile oysters, a significant interaction effect of temperature, salinity, and time ($F_{54, 280}$ = 9.715 p < 0.001) (Table S4) was observed, with low survival at salinities <10 for both temperature treatments (Fig. 4). Juvenile oysters tolerated salinities ≤5 for up to 4 d at 25°C but experienced significant mortalities (98.3 ± 2.9%) after 14 d (Fig. 4). During exposure to high temperatures (30°C), juvenile oysters suffered high mortality rates within 4 d at salinities <15 and 100% mortality by Day 6 at salinities ≤10 (Fig. 4). Salinities of 20–35 were favorable for survival of juvenile oysters, with ≥98% survival at 25°C and ≥82% survival at 30°C (Fig. 4, Table 1).

When juvenile oysters were exposed to gradually changing salinity (2–3/2 d), there was a significant interaction effect of temperature, salinity, and time on survival ($F_{135, 640}$ = 1.339; p = 0.004) (Table S5). By the time the salinity treatment of 5 was reached on Day 16, 81.7% (±1.7) survival was observed at 25°C and only 36.7% (±1.7) survival at 30°C, both of which continued to decrease resulting in a final survival of 21.7% (±1.7) and 0%, respectively (Fig. 5). In contrast to the acute change, a greater tolerance to salinities as low as 10 was observed with 41.7% (±1.7) and 26.7% (±1.7) survival at 25 and 30°C, respectively. High survival was observed when exposed to a salinity of 15 at 25°C (86.7 ± 1.7%), but at 30°C only 43.3% (±7.3) survival was observed. Similar to acute salinity change experiments, juvenile oysters generally experienced higher survival at 25°C compared to 30°C, even at moderate salinities (Fig. 5, Table 1).

3.3. Adult exposure

After 25 d of exposure, survival of adult oysters was significantly reduced during exposure to low salinities ($F_{4, 18}$ = 337.1; p ≤ 0.001) (Table S6). Survival in the 7 continuous and 7/14 cycle salinity treatments

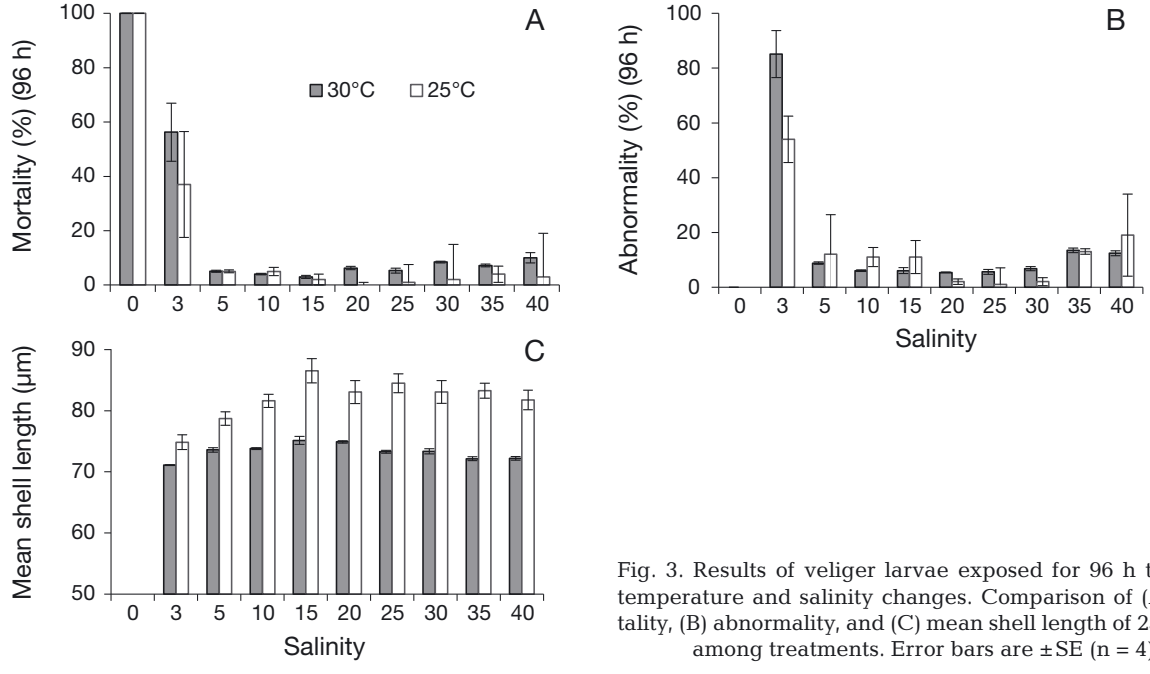

Fig. 3. Results of veliger larvae exposed for 96 h to acute temperature and salinity changes. Comparison of (A) mortality, (B) abnormality, and (C) mean shell length of 25 larvae among treatments. Error bars are ±SE (n = 4)

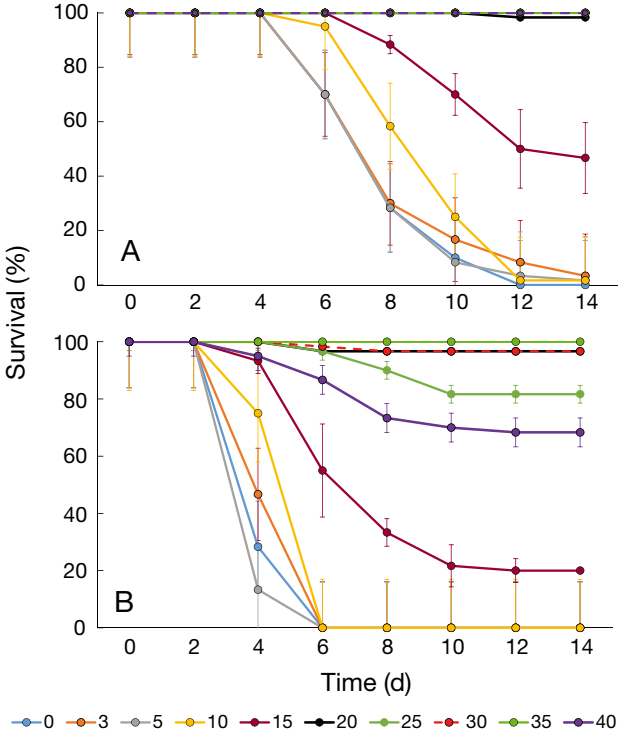

Fig. 4. Survival of juvenile oysters over time during exposure to acute salinity changes at (A) 25°C and (B) 30°C. Error bars are ± SE (n = 3)

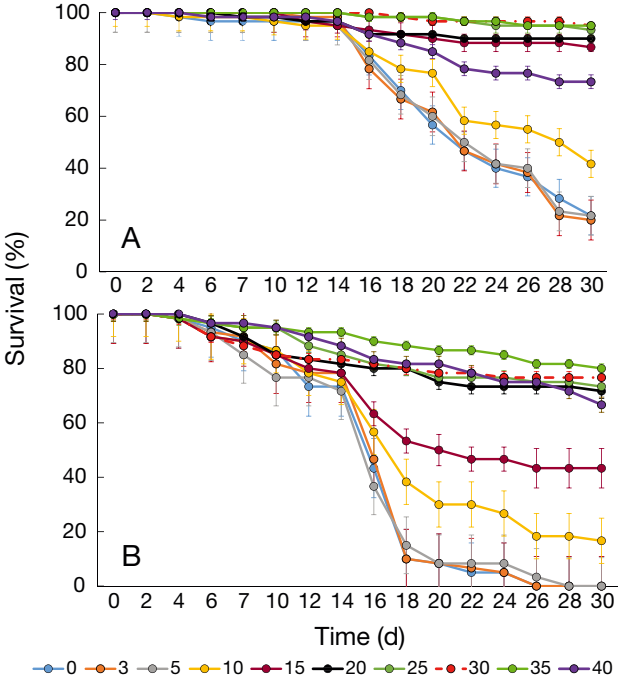

Fig. 5. Survival of juvenile oysters over time during exposure to gradual salinity changes at (A) 25°C and (B) 30°C. Final salinity was reached by Day 20 for all treatment salinities, and a salinity of 15 was reached by Day 12. Error bars are ± SE (n = 3)

Table 1. Mean survival time (d; Kaplan Meier Survival Analysis) of spat following exposure to acute and gradual salinity changes for both temperature treatments. To reduce artificial inflation during the gradual salinity adjustment period, Day 12 was treated as Day 0, when a salinity of 15 (the lower limit in the optimal range) was achieved. The exposure duration for acute and gradual exposures were 14 and 30 d, respectively

	Acute change		Gradual change	
	25°C	30°C	25°C	30°C
0	11.0	9.3	25.8	22.7
3	11.1	9.5	26.0	22.8
5	11.0	9.1	26.0	22.5
10	11.7	9.7	26.8	23.7
15	13.0	11.0	28.8	24.7
20	14.0	13.8	29.1	26.7
25	14.0	13.4	29.7	27.2
30	14.0	14.0	29.7	26.8
35	14.0	14.0	29.7	28.4
40	14.0	12.8	28.1	27.4

was significantly lower (82 and 87%, respectively) than the control (99%) (Tukey's HSD; $p \leq 0.05$), but no significant difference was observed between the 7 continuous and 7/14 cycle salinity treatments. Both salinity treatments of 3, continuous and 3/7 cycle, were significantly lower than all other treatments (Tukey's HSD, $p < 0.05$). Survival during continuous exposure to a salinity of 3 was significantly lower than the 3/10 cycle treatment (Tukey's HSD, $p < 0.05$), experiencing 100% mortality after 21 d of exposure (Fig. 6) compared to 85% mortality at the end of the experimental period in the 3/7 cycle salinity treatment. Adult oyster condition index also varied significantly among treatments ($F_{4, 294} = 43.925$; $p \leq 0.001$) (Table S7). There was no significant change in condition index over time in the control, but all treatment conditions were significantly lower than that of the control, and the 3/10 salinity cycle was significantly lower than all other treatments (Fig. 7) (Tukey's HSD, $p < 0.05$). No condition index is reported for continuous exposure to salinity treatment of 3 as there were no surviving oysters in this treatment at the end of the exposure.

4. DISCUSSION

A critical threshold salinity of 15 was observed for early life stages produced from moderately high salinity (25) broodstock oysters, and there was a synergistic effect of increased temperature at both low and high salinities (<15 and >35). Salinities of 15–30 in the summer months, when spawning is at a peak,

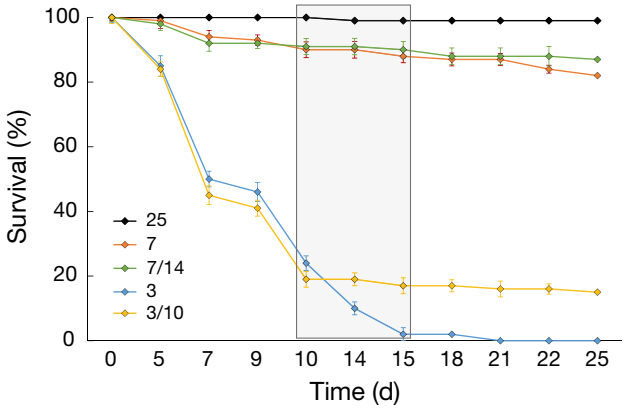

Fig. 6. Survival of adult oysters over time during 25 d of exposure to low salinity treatments. Salinities were elevated to 10 and 14 from 3 and 7, respectively, in the cycled salinity treatment from Days 10 to 15 (represented by the shaded box) and then returned to their initial salinities of 3 or 7, respectively, for the remainder of the exposure period. Error bars are ± SE (n = 5)

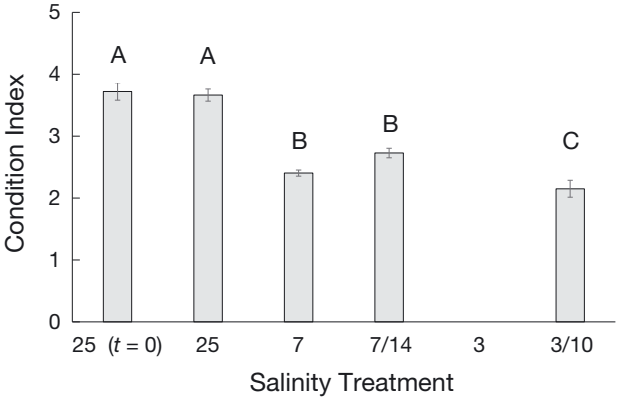

Fig. 7. Mean condition index for each salinity treatment following 25 d of exposure. Error bars are ±SE (n = 5). No survival was observed in the continuous treatment at a salinity of 3; therefore, no condition index is reported. Different letters represent significant differences among treatments (Tukey's HSD post hoc $p < 0.001$). $t = 0$ is time 0

would benefit all life stages, but adults were robust to low salinity events as low as 7 when temperatures were moderate (25°C). These 2 salinity limits represent summer and winter/spring salinity thresholds for moderately high salinity oyster populations (>20) and, where possible, can inform water management and site selection for restoration or aquaculture activities. The present study supports salinity tolerances previously reported in the literature and contributes to the existing body of work as it included the assessment across a wide range of salinities (0–40) across all life stages of the eastern oyster. This work also highlights the importance of timing (high and moderate temperatures) and rate of change (acute vs. gradual and continuous vs. fluctuating) in predicting resiliency of oysters to changes in salinity.

4.1. Early life stages

Gametes and pelagic life stages (embryos and larvae) lack the strong, structural safety of the developed shell, making them particularly vulnerable to changes in the external environment, and even for shelled larvae, prolonged valve closure increases the risk of sinking into the sediments and thus serves only as a short-term protection mechanism (Kennedy 1996, Thompson et al. 1996). It is therefore not surprising that we observed increased sensitivity to thermal and osmotic stress at earlier stages of development (gamete > embryo > D-hinge > spat). During acute exposure to salinities ≤10, fertilization was inhibited and hatch rates were low for gamete and embryo exposures, respectively. D-hinge larvae survived salinities down to 5, but growth was significantly reduced at salinities <15, suggesting that even though oyster larvae can survive, physiological impairments may result in long-term effects. Reduced feeding is a common response to salinity outside the optimal range and can lead to energetic deficiencies (Navarro 1988, McFarland et al. 2013, Lavaud et al. 2017), which could partially explain the observed reduction in growth. If conditions persist, the depletion of energetic reserves to fuel somatic maintenance, due to a lack of sufficient feeding, can ultimately result in death (Kooijman 2010).

The larval stage lasts approximately 2–3 wk (Kennedy 1996), but exposure during this study only persisted for 4 d. Thus, the effects may be amplified if the same low salinity conditions were to persist. Previous work has reported *Crassostrea virginica* larvae to be sensitive to salinities >35 but to tolerate salinities down to 7 (Davis 1958). Additionally, salinities ≥15 are reported as a requirement for growth and normal behavior when produced from high salinity broodstock (Chanley 1958, Davis 1958, Davis & Calabrese 1964). Calabrese & Davis (1970) observed low survival in *C. virginica* when exposed to salinities ≤12.5 during embryogenesis; however, exposure post-hatch resulted in successful metamorphosis at salinities as low as 7.5, although growth and survival rates were low compared to controls at 27.5. More recently, field observations of gametogenesis and juvenile recruitment in low salinity portions of the Hudson River Estuary, New York, have suggested that embryogenesis and larval development are possible in salinities as low as 6–10 (McFarland & Hare

2018). Scharping et al. (2019), found the optimal salinity range for successful embryogenesis to be 7–16, when produced from mesohaline broodstock conditioned at a salinity of 11. The discrepencies between studies may be largely due to previous life history and parental exposure to low salinities (Loosanoff 1952, Davis 1958, Davis & Calabrese 1964, Devakie & Ali 2000, Eierman & Hare 2013). During the present study, broodstock were conditioned and spawned at a salinity of 25, making a salinity drop to ≤10 or an increase to 40 quite dramatic for a developing embryo or free gamete with limited physical protection against osmotic shock (Carriker 1996). Eierman & Hare (2013) highlight the importance of broodstock exposure in determining the range at which progeny can thrive using reciprocol transplants. The salinity ranges reported in this study should therefore be interpreted with reference to moderately high salinity oyster populations.

For 1 mo old spat, high mortality rates were observed at salinities ≤10 in response to both gradual and acute changes. Spat were, however, able to tolerate salinities as low as 0 for up to 4 d, showing resiliency if exposure is brief. This short-term survival to extreme low salinity is likely due to valve closure and seclusion from the environment (Davenport 1979, Carriker 1996, Berger & Kharazova 1997). Valve closure acts as a barrier to allow for the maintenance of internal salt concentrations above that of the external environment for several days (La Peyre et al. 2003, 2013, McFarland et al. 2013). However, valve closure also prevents feeding and the exchange of gases, forcing the animal into anaerobic respiration and, thus, cannot be maintained indefinitely (Berger & Kharazova 1997). High temperatures exacerbate the negative effects of environmental perturbation due to increased metabolic rates (Bougrier et al. 1995), resulting in an accumulation of waste products, limiting the duration of valve closure (Michaelidis et al. 2005, La Peyre et al. 2013). This biological response is the likely explanation for the higher mortality rates observed at 30°C, compared to 25°C.

The acute temperature increase amplified the impact of salinity extremes for all early life stages. While temperatures of ≥30°C are common in southern US estuaries, the aim of this study was to induce both a thermal and osmotic stress response, and therefore the change in temperature was acute, moving gametes, embryos, larvae, and spat directly from 25 to 30°C. When this acute thermal stress is then coupled with osmotic stress, we saw a reduced tolerance to salinities outside the optimal range for polyhaline oyster populations (salinities of 14–28; Shumway 1996). An interesting point of comparison is survival of spat for both temperatures and rates of change at a salinity of 15 (Table 1), which appears to be an important threshold salinity for juvenile oysters. A lower temperature (25°C) and a gradual change allowed for a wider salinity tolerance, across all treatments; however, 15 was the salinity at which mortality rates deviated from the controls and below which mortality rate accelerated. While 15 is within the expected optimal salinity range for *C. virginica* (Shumway 1996), elevated mortality was observed when temperature was also high, even when the salinity change was gradual. High survival (87%) was observed at a salinity of 15 when the change was gradual at a moderate temperature (25°C); however, the combined acute salinity decrease (by 10) and acute temperature increase (by 5°C) proved to be fatal for spat, resulting in only 20% survival for salinity 15 exposure at 30°C. Gradual changes in salinity allow for the oyster to slowly adjust internal body fluids to the external environment, while an acute change can cause osmotic shock and often irreparable cellular damage (Rivera-Ingraham & Lingot 2017) and, therefore, higher mortality rates. Similarly, a change in temperature by 5°C adds physiological stress, and increased metabolic demands when temperatures are elevated can lead to increased mortality due energy imbalance (Bougrier et al. 1995, Kooijman 2010). Given the short timescale of these exposures, an acute temperature change was required to achieve the intended salinity by temperature response. It is plausible to reason that a gradual change in temperature would expand the salinity range tolerated at the elevated temperature and warrants further investigation to understand the long-term effects of climate change on the eastern oyster.

4.2. Adult exposure to fluctuating salinity

Adult oysters had the greatest tolerance to low salinity events of all life stages with high survival rates at a salinity of 7; however, reduced condition index suggests physiological impairment that could have lethal effects if conditions persist (Andrews et al. 1959, La Peyre et al. 2009). The reduction in condition index at low salinities could be due in part to inhibition of feeding during valve closure in response to low salinity conditions, the reabsorption of gametes, or both (Lavaud et al. 2017, Casas et al. 2018b), leading to a 'shrinking' of somatic tissue (Kooijman 2010). When experiencing osmotic stress, energy expenditure is often elevated to maintain cellular protection

and osmotic homeostasis (Sokolova et al. 2012, Rivera-Ingraham & Lingot 2017). This coupled with cessation of feeding during hermitization from the external environment leads to an energy imbalance (Heilmayer et al. 2008), which is amplified under elevated temperatures due to increased metabolic rates (Bougrier et al. 1995). In addition, reproductive state plays a role in the ability of the individual to tolerate environmental perturbations and can result in mortality due to the high metabolic demand of gametogenesis (Andrews et al. 1959, Wendling & Wegner 2013, Miller et al. 2014). In this study, adult oysters were collected in early spring (late March 2014), when temperatures were moderate (~24°C) and gametogenesis was expected to be in the early stages for southwest Florida (Volety et al. 2009, McFarland et al. 2022). Adult oysters may be more sensitive to acute changes in the summer months due to the added metabolic stress of late stage gametogenesis and spawning coupled with higher temperatures (La Peyre et al. 2003, McCarty et al. 2020).

When salinity was held at 3, mortality in adult oysters was low during the first 5 d but accelerated rapidly thereafter, suggesting a 5 d maximum for extreme low salinity events at moderate temperatures (25°C). Adult mortality continued on this trajectory for the continuous treatment at a salinity of 3, but in the 3/7 cycle treatment, mortality leveled off during and immediately following the 5 d reprieve when salinity was raised from 3 to 7, suggesting that the short-term reprieve allowed for some physiological adjustment to the low salinity environment. More alarming is that this experiment was conducted at 25°C, and, based on spat and early life stage exposures, it can be predicted that adult mortality would be amplified if temperature was also elevated. In southern regions, temperatures ≥30°C are common in the summer months when salinities are also at their seasonal low (McFarland et al. 2022), increasing the threat to existing populations. McFarland et al. (2022) reported significant mortality events in the broodstock populations when low salinity exposure occurred in the late summer, but these same populations tolerated prolonged low salinity (<5) during the winter and spring when temperature was low (≤20°C). Previous laboratory studies have also shown adult oysters to be more tolerant of low salinity events in the winter and spring, but the same populations experience elevated mortality rates if the exposure occurs in the summer months (La Peyre et al. 2003, McCarty et al. 2020). This highlights the importance of seasonal timing and the physiological state of the oysters when understanding tolerance to extreme events.

4.3. Ecological implications

Salinity is a major factor dictating *C. virginica* reef density and distribution (Shumway 1996), and while *C. virginica* populations are capable of thriving under a wide range of salinity and temperature fluctuations, regional differences in tolerance suggest that plasticity may have its limits. Whether this is due specifically to previous exposure and acclimation or if there are underlying genetic changes that occur due to isolation by distance remains to be fully understood (but see Eierman & Hare 2013, 2016). Oysters have shown resilience to extreme low salinities (<5) during winter and spring, but these same salinity extremes are lethal during the summer months when temperatures are high and gametogenesis is at a peak (La Peyre et al. 2003, 2009, 2013, Levinton et al. 2011, McFarland et al. 2022). This work has shown that even short exposure (e.g. 4 d) to salinity extremes can be devastating during the spawning season when pelagic life stages and recently settled spat are most vulnerable. Understanding how salinity and temperature interactions affect oysters at all life stages is particularly important in highly managed estuaries, where the timing of controlled freshwater releases may either amplify or reduce negative effects of osmotic stress on oysters of all life stages (McFarland et al. 2022), and to inform aquaculture site selection (Swam et al. 2022).

Where freshwater flow can be managed, the timing of large magnitude freshets should aim to avoid periods when elevated temperatures put all life stages at risk (La Peyre et al. 2003, 2009, McFarland et al. 2022) by limiting high volume freshwater releases to periods in which oysters are not reproductively active and larvae are not expected to be in the water column (winter and early spring). To be most effective, short duration exposure to low salinity events (e.g. 7 for up to 25 d or 3 for up to 5 d) in the winter or early spring would not only be safe to the oyster population, but it may provide a beneficial reprieve from disease and predation pressures (La Peyre et al. 2003, 2009). Global climate change predictions suggest rising temperatures along with an intensification of El Niño Southern Oscillations (Fasullo et al. 2018) and storm events (Elsner et al. 2008), 2 factors that can cause rapid and extreme changes in salinity. It is therefore critical that seasonal timing and temperature are taken into consideration when predicting response to variation in salinity and forecasting impacts on oyster populations. Our work has shown the vulnerability of all life stages to the synergistic impacts of thermal and osmotic stress.

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