Timing of stressors alters interactive effects on a coastal foundation species

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Abstract. The effects of climate-driven stressors on organismal performance and ecosystem functioning have been investigated across many systems; however, manipulative experiments generally apply stressors as constant and simultaneous treatments, rather than accurately reflecting temporal patterns in the natural environment. Here, we assessed the effects of temporal patterns of high aerial temperature and low salinity on survival of Olympia oysters (*Ostrea lurida*), a foundation species of conservation and restoration concern. As single stressors, low salinity (5 and 10 psu) and the highest air temperature (40°C) resulted in oyster mortality of 55.8, 11.3, and 23.5%, respectively. When applied on the same day, low salinity and high air temperature had synergistic negative effects that increased oyster mortality. This was true even for stressor levels that were relatively mild when applied alone (10 psu and 35°C). However, recovery times of two or four weeks between stressors eliminated the synergistic effects. Given that most natural systems threatened by climate change are subject to multiple stressors that vary in the timing of their occurrence, our results suggest that it is important to examine temporal variation of stressors in order to more accurately understand the possible biological responses to global change.

Key words: aerial temperature; climate change; extreme events; multiple stressors; Olympia oyster; Ostrea lurida; salinity; synergistic; temporal patterns.

Introduction

Many anthropogenic stressors pose substantial threats to natural systems (Sala et al. 2000, Hoegh-Guldberg and Bruno 2010). Stressors associated with climate change are particularly widespread (Walther et al. 2002, Parmesan and Yohe 2003, Tylianakis et al. 2008, Bellard et al. 2012, Doney et al. 2012) and include increasing temperatures, rising sea levels, changes in seawater chemistry and salinity, and increases in extreme events such as heat waves and storms (Solomon et al. 2009, IPCC 2014). Multiple stressors can have interactive effects, where the combined effect is less than (antagonistic) or greater than (synergistic) the expectations based on the individual effects alone (Breitburg and Riedel 2005, Crain et al. 2008). Such interactions mean that studies of single stressors may not accurately predict responses to multiple stressors (Klug and Cottingham 2001, Darling and Côté 2008, Breitburg et al. 2009, Todgham and Stillman 2013). Most multiple stressor

studies have been conducted with constant and simultaneous stressors that may not reflect the temporal patterns often present in nature (Davenport 1982, Crain et al. 2008, Przeslawski et al. 2015, for exceptions, see García Molinos and Donohue 2010, Pincebourde et al. 2012, Cheng et al. 2015). Thus, there is a strong need to increase realism in climate change studies (Wernberg et al. 2012) and specifically to understand how temporal variation in stressors will affect species and ecosystems (Denny et al. 2009, Lawson et al. 2015). Moreover, understanding this temporal variation is critical for conservation, management, and policy, because of projected increases in environmental variation due to climate change (Fischer et al. 2013, IPCC 2014).

Stressor timing can vary in several key ways, including fluctuations in individual stressors over time. Many studies have focused on changes in mean climate conditions (Easterling et al. 2000, Smith 2011), but fluctuations around mean conditions can profoundly influence how organisms respond to stressors (Lawson et al. 2015, Ma et al. 2015). In particular, extreme events can influence ecological dynamics more than the mean level of a given factor (Gaines and Denny 1993, Easterling et al. 2000, Denny et al. 2009) and can play a critical role in

biotic responses to climatic shifts (Stenseth et al. 2002, Helmuth et al. 2006, Zimmermann et al. 2009). Given the ecological importance of extreme events and their increased likelihood due to climate change (Coumou and Rahmstorf 2012), it is important to incorporate extreme stressor levels in studies of potential climate change impacts (Smith 2011).

In addition to variation within a single stressor, stressors vary in their timing relative to one another. Multiple stressors can occur simultaneously or nearly simultaneously (i.e., coincidentally), consecutively (i.e., sequentially), or decoupled in time. Most previous studies have focused on coincident multiple stressors, perhaps for ease of experimentation or because simultaneous stressors are often expected to result in the most detrimental effects in marine systems (Denny et al. 2009). Indeed, although multiple stressors are often additive or antagonistic in terrestrial systems (Côté et al. 2016), and antagonistic in freshwater systems (Jackson et al. 2016), meta-analyses of multiple anthropogenic stressors in marine systems often revealed synergistic effects (Burkepile and Hay 2006, Crain et al. 2008, Harvey et al. 2013, Przeslawski et al. 2015). This suggests that effects from coincident stressors in the marine environment may often be more severe than predicted based on effects from single stressors. However, simultaneous stressors do not always pose the greatest threat (García Molinos and Donohue 2010). As examples, consecutive patterns of water and air temperature stress were most disadvantageous for sea stars, resulting in the lowest feeding rates (Pincebourde et al. 2012), and sequential applications of acidified conditions and salinity affected oxygen consumption of crab larvae more than simultaneous application of those stressors (Miller et al. 2014). Consecutive stressors can be particularly detrimental because organisms, although only exposed to one stressor at a time, are stressed for a longer duration without any recovery time (Pincebourde et al. 2012). Additionally, exposure to one stressor can trigger physiological changes that may make organisms more robust against that stressor, but potentially also more vulnerable to a different stressor (Todgham and Stillman 2013). Complete decoupling of stressors might be predicted to lessen negative effects. For example, an ecologically realistic delay between initial warming and hypoxia and subsequent salinity stress resulted in additive, independent effects on oyster growth and survival (Cheng et al. 2015).

Intertidal ecosystems are excellent model systems for assessing temporal patterns of multiple stressors; they are physiologically challenging habitats in which organisms must contend with both terrestrial and marine stressors that can interact with varying temporal patterns (Somero 2002). In particular, intertidal estuarine organisms are often challenged by fluctuating air temperatures and salinity levels. One example is the Olympia oyster (*Ostrea lurida*), an important foundation species and the only native oyster on the west coast of the United States and Canada. These animals are relatively heat

tolerant with an LT50 of approximately 38°C in seawater (Brown et al. 2004, Cheng et al. 2017) and are capable of withstanding low salinities for short durations (Cheng et al. 2017). However, projected increases in the frequency and magnitude of extreme precipitation events and heat waves may pose substantial threats to Olympia oysters (Cheng et al. 2016). Although seawater temperatures are not expected to approach 38°C, intertidal air temperatures can already exceed this level (Fig. 1) and heat waves are expected to increase in frequency and severity with climate change (IPCC, 2014). Other studies have addressed impacts of high water temperatures on Olympia oysters (Brown et al. 2004, Cheng et al. 2017), but impacts of high air temperatures have yet to be examined. Organisms respond differently to air and water temperatures (Jones et al. 2009, Yamane and Gilman 2009, Pincebourde et al. 2012, Dowd and Somero 2013), and thus, it is important to test their responses in the environment in which they will realistically
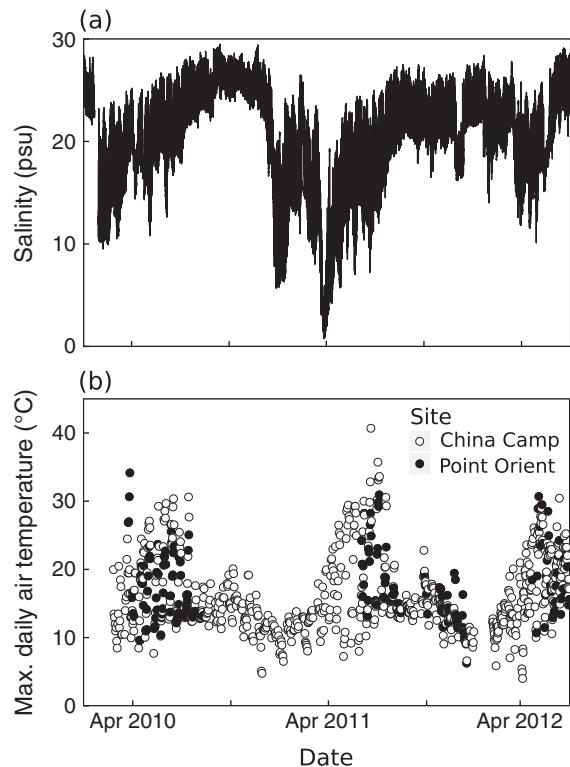

FIG. 1. Field conditions at China Camp State Park and Point Orient, San Francisco Bay, California, USA (see Appendix S1: Table S1 for locations), which were used to set experimental conditions. (a) Salinity at China Camp from the National Estuarine Research Reserve System-wide Monitoring Program (NERRS, 2015) and (b) maximum daily air temperature from intertidal loggers at China Camp and Point Orient (attached with cable ties to rebar stakes approximately 15 cm above the sediment at 0.0 m mean lower low water). High air temperature can occur in the spring (2010 at Point Orient) or in the summer (2011 at China Camp). Spring low salinity can coincide with high air temperature (e.g., 2010 at Point Orient) or can precede summer high temperatures (e.g., 2011 at China Camp).

experience a given temperature (Bjelde and Todgham 2013). Rising global temperatures are also expected to result in higher rates of evaporation and a nearly exponential increase in atmospheric water-holding capacity, leading to heavier precipitation and more intense flood events associated with a strengthened El Niño Southern Oscillation (Min et al. 2011, Das et al. 2013, Yoon et al. 2015). Increased precipitation can lead to extreme low salinity events in estuaries (e.g., 5 psu for 8 d; Fig. 1) and cause oyster mortality (Cheng et al. 2016).

Here, we tested for interactive effects of varying levels of low salinity and high air temperature, including extreme levels, and their temporal patterning on Olympia oyster survival. We performed a laboratory experiment to answer the following questions: (1) Is there an interactive effect of low salinity and high air temperature? (2) How does the interaction between these two stressors differ when they are applied in the same time period vs. when they are decoupled in time (low salinity followed by high air temperature)? Although previous meta-analyses showed that stressful levels of salinity and water temperature often produce antagonistic effects on a variety of marine taxa (Crain et al. 2008), low salinity and high air temperature can combine to have synergistic effects on intertidal organisms (Williams et al. 2011). Both emersion and warm temperatures can lead to oxygen debt and a high metabolic demand, requiring increased oxygen uptake upon submergence (McMahon 1988, Somero 2002). If submergence occurs in low salinity seawater after exposure to hot air, oysters would need to open their valves to gain access to oxygen, but also seal their valves to prevent osmotic stress, because they do not actively osmoregulate (Shumway 1977). These opposing needs are physiologically incompatible. Thus, we hypothesized that these two stressors would cause different, but related effects, and that oysters exposed to high air temperature and low salinity on the same days would suffer increased mortality as a result of synergistic effects. We hypothesized that oysters given two weeks recovery time (to mimic a submersion between two tide series) after an initial salinity stress and before a subsequent air temperature stress would also suffer greater mortality due to synergistic effects associated with negative long-term consequences of osmotic stress. We expected the magnitude of this synergistic effect to be less severe than with the coincident treatment. When given four weeks between stressors, we hypothesized that oysters would be fully recovered from their initial stress and salinity and temperature would act independently.

MATERIALS AND METHODS

General approach

To assess the influence of temporal patterns of multiple stressors on the survival of Olympia oysters, we performed a laboratory experiment at the Bodega Marine Laboratory, University of California, Davis in winter 2013. We collected adult Olympia oysters ($n \approx 100$ per site) from the low intertidal zone at approximately 0.0 m mean lower low water at six sites distributed throughout San Francisco Bay (Appendix S1: Table S1) in July 2013. We spawned the wild broodstock and then combined the larvae from our six sites to achieve a representative sample from San Francisco Bay. We transferred the larvae into 100 L culturing cylinders that were lined with 10×10 cm PVC tiles for settlement (Cheng et al. 2015). We raised offspring under common laboratory conditions, and then subjected the juvenile oysters to experimental conditions (for oyster husbandry details see Appendix S2: Text S1). To investigate the potential interaction of low salinity and high temperature, we assessed survival of a subset of juvenile oysters that experienced both stressors coincidentally. To assess the importance of temporal patterns of stressors, we analyzed survival of oysters that were given different amounts of recovery time between the two stressors.

Environmental stressors

To simulate current and potential future exposures, we based our environmental stressor levels and durations on field data collected from San Francisco Bay (Fig. 1) and on projections for future changes. San Francisco Bay is an excellent system for testing the effects of these two stressors. It has a Mediterranean climate where summers are dry and winters tend to be wet. Additionally, San Francisco Bay receives run-off from approximately 40% of California (Conomos 1979), which can result in low salinity events during sustained winter storms and spring snow melt. Heat waves that can occur in the spring, when low tides are mid-day and the cooling influence of summer fog is minimized (Null 1995), are stressful for intertidal systems (Mislan et al. 2009). These two stressors could co-occur in the spring under extreme conditions, or could be decoupled if low salinity occurs in the winter and high air temperature occurs in the late spring or summer (Fig. 1). Additionally, these two stressors co-occur spatially in the northern portion of the estuary where freshwater enters and the cooling influence of the ocean is diminished (Mislan et al. 2009, Wasson et al. 2014). Downscaled climate models project increased air temperatures and more frequent extreme precipitation events in the San Francisco Bay region (Cloern et al. 2011, Yoon et al. 2015).

We used 4-month old oysters, the earliest age at which they would experience low salinity and high air temperature in San Francisco Bay given peak oyster settlement in the late summer and fall (Chang et al. 2016) and stressor presence in the winter and spring. The juvenile life stage can be particularly sensitive to physiological stress (Bible and Sanford 2016), with mortality rates that sometimes exceed those for larvae (Gosselin and Qian 1997, Hunt and Scheibling 1997). Thus, we assessed stressor effects on the vulnerable juvenile stage and at a time when they would realistically experience these stressors.

To determine whether there was an interactive effect of low salinity and high air temperature, we assessed oyster survival after exposure to nine treatment combinations composed of three salinity levels (33, 10, and 5 psu) and three air temperature levels (18, 35, and 40°C). The control salinity level (33 psu) represents full strength seawater, an ambient level for oysters in some locations and a level that has been used in previous studies as a nonstressful, control condition (Cheng et al. 2015). The two lower salinity levels represent levels that occur during seasonal low salinity events (Fig. 1a). The most extreme low salinity level (5 psu) is experienced occasionally in northern San Francisco Bay (Cheng et al. 2015), and may increase in frequency with extreme precipitation and flooding associated with climate change (Cloern et al. 2011, Min et al. 2011, Das et al. 2013, Yoon et al. 2015). Salinity treatments were applied as constant stressors over a period of 7 d to mimic the duration of extreme low salinity events that have recently occurred in northern San Francisco Bay (Fig. 1a) and because previous experiments found this duration to be near the threshold for oyster mortality (Cheng et al. 2015, Bible and Sanford 2016). The control air temperature (18°C) reflects a common daily high air temperature at our sites around San Francisco Bay (Appendix S1: Table S1) during winter and spring. The 35°C treatment reflects a high temperature that occurs periodically and the 40°C treatment reflects the highest temperature we recorded at any site from 2010 to 2012 (Fig. 1b) and represents a stressor level that can be expected more frequently with climate change (Cloern et al. 2011, IPCC 2014). Additionally, initial trials found 40°C was a threshold for oyster mortality (Appendix S3: Figs. S1, S2). Aerial exposure was applied as a variable stressor to mimic the realistic heating and cooling that is driven by exposure at low tide and inundation at high tide as well as changes in air temperature over the course of a low tide. Realistic rates of increase and decrease for each experimental temperature were determined from San Francisco Bay field data (Appendix S3: Fig. S3). To simulate a multi-day heat wave during a spring tide series at 0.0 m mean lower low water (a tidal elevation where oysters are common) an aerial stressor event in this experiment involved 3.5 h of exposure, repeated 3 d in a row. Aerial exposure in the lab approximated oyster temperature in the field, where other factors including conduction and evaporation would influence body temperature (Helmuth 1998).

In addition to assessing coincident application of salinity and temperature stressors, we manipulated the timing of these stressors relative to one another, with an initial salinity challenge followed by a high air temperature event. We used three timing treatments: coincident (stressors applied on the same days), decoupled by two weeks, and decoupled by four weeks. Our coincident treatment mimics extreme spring conditions, when oysters could theoretically experience low salinity and high air temperature stress on the same days. Our decoupling treatments represent more common scenarios when low salinity stress occurs first in the winter and is followed by a low tide during a period of high air temperatures in the spring (Fig. 1).

Experimental design

Prior to the experiment, oysters were held at 33 psu. To acclimate oysters to aerial exposure, starting one month prior to the experiment, oysters were exposed to air (17.8–18.1°C) for 4 h, 3 d in a row, every 2 weeks to mimic exposure during low tides. We assigned tiles to treatment combinations of salinity, temperature, and timing (Fig. 2; three salinities × three temperatures with coincident timing = nine combinations; three salinities × two temperatures × two timings for decoupled tests = 12 combinations; 21 combinations total). Assignments were made using stratified randomization based on the number of oysters per tile (10–20 oysters/tile; 12 tiles (experimental units) per treatment combination = 252 tiles, mean of 193 oysters per treatment combination = 4053 total oysters at start of experiment. Note that analysis was performed on 234 tiles, 3,776 oysters (see *Statistical analyses*)). There were six replicate 38 L tanks for each salinity level (six tanks × three levels = 18 tanks). Salinity tanks functioned as blocks; within each tank there were two tiles from each temperature × timing combination for a total of 14 tiles per salinity tank (Appendix S3: Fig. S4). Individual tiles were removed from each tank and taken to their respective incubators for each aerial exposure. There was one incubator for each of the three temperatures. Starting one week before and continuing throughout the experiment, oysters were held in a temperature-controlled room (mean ± SD = 11.33 ± 0.85°C) to mimic water temperatures during late winter/early spring when low salinity would occur in the field (Cheng et al. 2015). For additional oyster husbandry details see Appendix S2: Text S1.

To assess the effects of coincident application of salinity and temperature, we exposed oysters to their salinity treatment (5, 10, or 33 psu) for 7 d and their air temperature treatment (18, 35, or 40°C) during the last 3 d of their salinity exposure (Fig. 2a). For oysters in the 5 and 10 psu treatments, salinity was decreased gradually from 33 psu over the course of 5 d and then all oysters were kept at target salinities, measured in each tank daily (mean ± SD = 33.04 ± 0.16 psu, 10.04 ± 0.13 psu, 5.05 ± 0.14 psu), for the seven-day exposure. Salinities were maintained during daily water changes by mixing seawater and distilled water to target salinities. On the fifth day of salinity exposure, all tiles were individually removed from their salinity treatments and transferred to incubators for their respective aerial exposure. Incubators (model 136VL, Percival Scientific, Inc., Perry, IA, USA) were pre-equilibrated to starting temperatures, ramped up to maximum temperatures (18, 35, or 40°C) over the course of 2 h, held at the maximum for 30 min, and then temperatures were decreased over the course of one hour (Appendix S3: Fig. S3). After 3.5 h in the
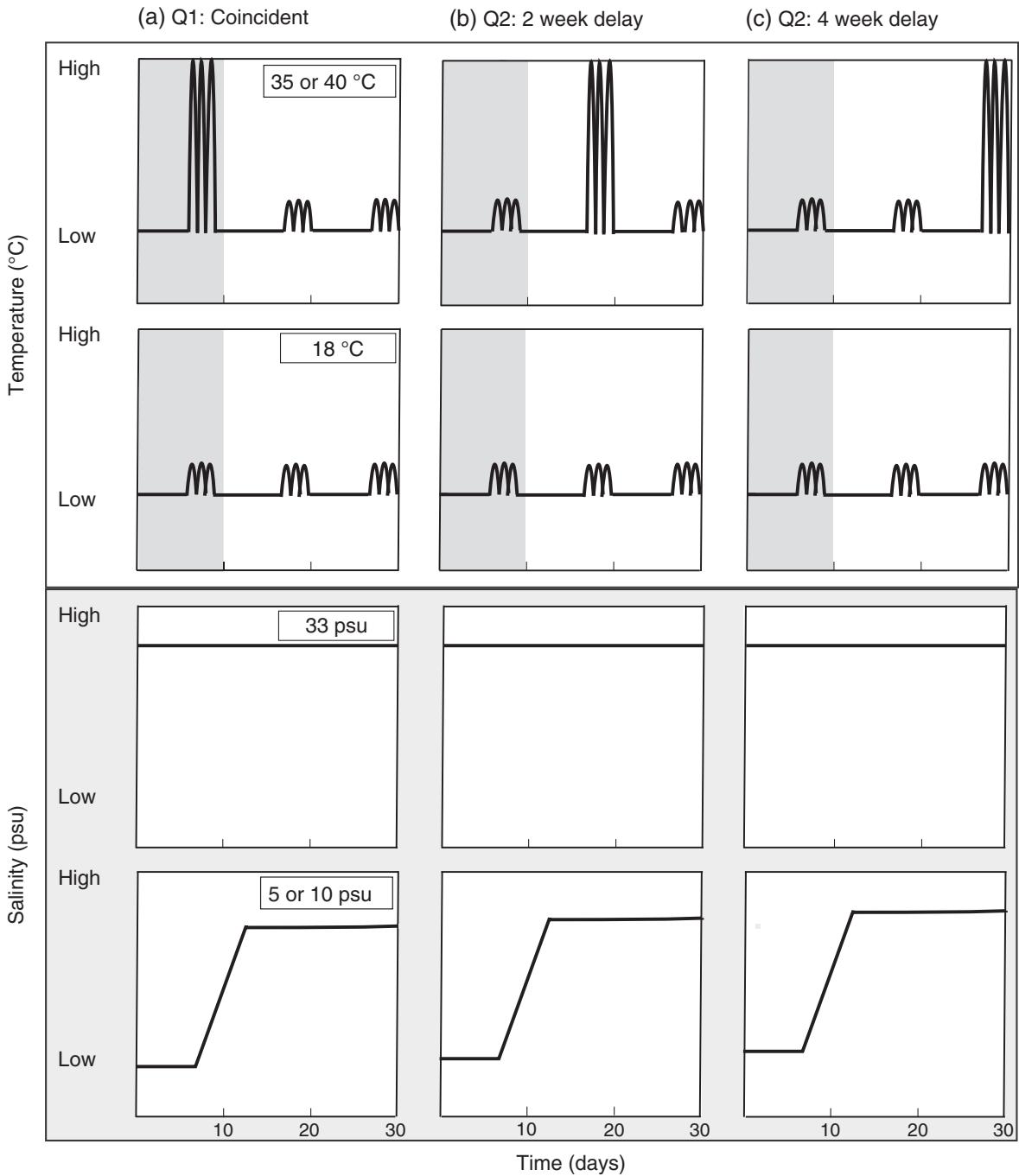
Fig. 2. Experimental protocol for factorial experiments. Each tile with settled oysters was assigned one salinity treatment (control level of 33 psu or one of two low salinity levels of 10 or 5 psu; lower two panels) that they experienced for a seven-day treatment. Each tile was also assigned one aerial temperature treatment (control level of 18°C or one of two high temperature levels of 35 or 40°C; upper two panels) that they experienced for 3.5 h, 3 d in a row during one of three timings relative to the salinity treatment (coincident, decoupled by 2 weeks, and decoupled by 4 weeks). The two upper panels show high peaks when oysters experienced high air temperature and low peaks when they experienced control air temperature. Each temperature was paired with each salinity. (a) Coincident salinity and air temperature stress. This design addresses interactive effects of these two stressors. (b) Salinity stress followed by two weeks recovery time before air temperature stress. (c) Salinity stress followed by four weeks recovery time before air temperature stress. Panels b-c address how the interaction changes with different timing of stressors. Gray boxes in top temperature panels indicate when salinity stress was applied.

incubators, we transferred individual tiles back to their respective salinity treatment tanks. Oysters were exposed to their assigned air temperature treatment (18, 35, or 40°C) for the final 3 d of the salinity treatment (Fig. 2a) (for additional aerial exposure methods see Appendix S2: Text S2). After those 3 d, we returned oysters to non-stressful conditions: full strength salinity (33 psu) and control water temperature (mean ± SD = 10.92 ± 0.97°C). Oysters assigned to coincident effects of salinity and air temperature were exposed to air again after 2 and 4 weeks of recovery time, but this exposure was always at the control temperature (18°C) (Fig. 2a).

To assess the effects of timing of stressors, a subset of oysters that experienced control air temperatures during the aerial exposure that coincided with the salinity treatment were exposed to a high aerial temperature at either two or four weeks after the salinity treatment (Fig. 2). All oysters experienced aerial exposure at all three times (coincident with salinity, and two and four weeks after salinity exposure), but oysters were only exposed to high temperatures during the period corresponding to their treatment (Fig. 2). Control oysters experienced 18°C for all three exposures. Just as with the first aerial exposure, all subsequent exposures consisted of 3.5 h in the incubators, for 3 d in a row. To make certain delayed mortality associated with these stressors was assessed, oysters were kept in non-stressful conditions for three weeks after the final aerial exposure before mortality was determined (Brown et al. 2004).

Statistical analyses

To assess the effects of low salinity, high temperature, and their timing on oyster survival, we used a generalized linear mixed model with binomial error distributions and logit link functions for each timing treatment. We used binary response data from individual oysters (dead = 0, live = 1) in a nested design where tile nested within tank and tank were random effects and salinity, air temperature, and their interaction were fixed effects. Model overdispersion was not possible because we used binary response data (i.e., a Bernoulli trial; McCullagh and Nelder 1989). We compared treatment effects using Wald χ^2 tests (Bolker et al. 2009) and multiple comparisons were carried out using general linear hypothesis tests. Analyses and graphics were produced using R (version 3.1.0) and the lme4, car, multcomp, brglm, and ggplot2 packages (Appendix S2: Text S3).

When stressors were coincident, the data exhibited complete separation. Complete separation occurs when there is zero variation within a treatment level (e.g., 100% oyster mortality), which can prevent model convergence or produce unreliable parameter estimates. We therefore added one survivor to the treatment group that experienced 5 psu and 40°C coincidentally, which led to 99.0% mortality, but allowed for successful modeling using a generalized linear mixed model. We also modeled the data using a bias-reduced method that allows

for completely separated data, but cannot incorporate random effects (Firth 1993). The two models produced virtually identical results so we continued with the glmm model that accounts for the nested design of our experiment. Initial visual analyses of the data revealed a tank effect in the 33 psu, 40°C treatment. Five tanks had survival percentages ranging from 79.2–92%, while the sixth tank had 2.2% survival. Because there was spatial and temporal variation in incubator temperature (Appendix S3: Fig. S5), we hypothesize that on this particular day, the part of the incubator this tank occupied reached a high enough temperature that nearly all oysters died. Because this high mortality was isolated to one tank, we removed that tank from the analyses, which reduced our experimental units from 252 to 234 tiles.

RESULTS

Oysters in control conditions (33 psu, 18°C) only experienced 3% mortality (Fig. 3a). As single stressors, both low salinities and the highest air temperature resulted in significant oyster mortality. When paired with
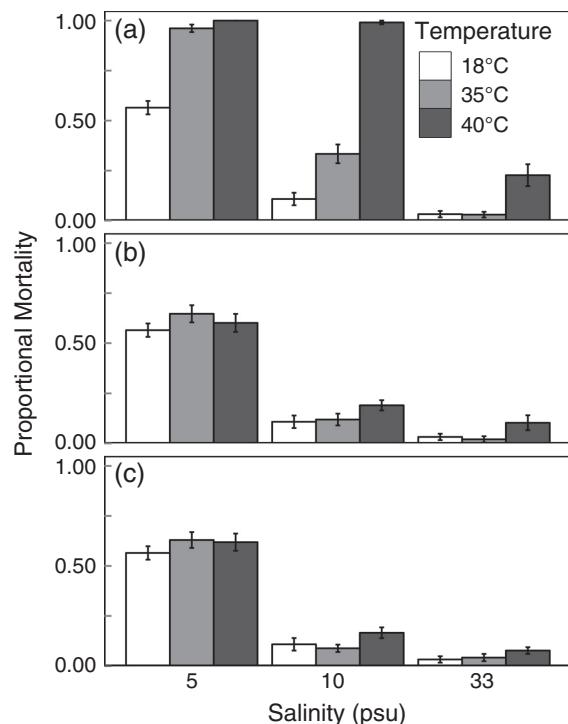

FIG. 3. Variation in mortality among oysters exposed to high temperature at different time intervals after salinity treatments: (a) 0 weeks, (b) 2 weeks, and (c) 4 weeks. Data from control oysters (18°C) are repeated in each panel for ease of visual comparison. Bars are mean proportional oyster mortality (± SEM) based on 6–12 tiles per treatment (234 tiles, 3776 oysters total). The coincident (0 weeks), 5 psu, 40°C treatment resulted in 100% mortality. Note that statistical analyses were based on individual oyster mortality in a generalized linear mixed model with a nested design. Data are presented here as proportional mortality for ease of visualization.

control air temperature (18°C), the lowest salinity (5 psu) resulted in 55.8% mortality ($Z = -7.009$, $P < 0.0001$) and intermediate salinity (10 psu) resulted in 11.3% mortality ($Z = -2.365$, $P = 0.047$). When paired with control salinity (33 psu), the highest air temperature (40°C treatment) resulted in 23.5% mortality ($Z = -3.694$, $P < 0.001$). Intermediate air temperature (35°C treatment) did not result in more mortality than control conditions (Appendix S1: Table S2).

When oysters experienced low salinity and high air temperature on the same days (coincident treatment), there were significant effects of salinity (Wald $\chi^2 = 216.4$, $P < 0.0001$) and air temperature (Wald $\chi^2 = 99.45$, $P < 0.0001$), and a significant synergistic interaction between them (Wald $\chi^2 = 30.99$, $P < 0.0001$). Here, we define synergistic effects as resulting in a statistical interaction and causing a larger effect (i.e., more mortality) than expected based on stressors applied individually. We determined the direction of the effect by looking at the sign of the interaction parameter estimates. Here, negative estimates indicate decreased survival or increased mortality as compared to expectations based on no interaction. All stressor levels, including those that were not lethal or had only mild effects when applied alone, showed synergistic effects when applied together (Fig 3a, Appendix S1: Table S3). At 10 psu, oysters exposed to 35°C suffered 31.6% mortality ($Z = -3.934$, $P < 0.001$) and those exposed to 40°C nearly all died (99.0% mortality; $Z = -6.388$, $P < 0.001$). At 5 psu, oysters exposed to 35°C experienced 95.9% mortality ($Z = -7.449$, $P < 0.001$) and at 40°C there was 100% mortality ($Z = -4.270$, $P < 0.001$); the difference between the two high temperatures at 5 psu was not statistically significant (Fig. 3a, Appendix S1: Table S2).

As opposed to the synergistic effects found when low salinity and high temperature were coincident, two week decoupling of stressors (Fig. 3b) resulted in only marginal evidence for an interaction and four week decoupling of stressors (Fig. 3c) resulted in no interaction. When stressors were decoupled by two weeks, we found significant effects of salinity (Wald $\chi^2 = 101.95$, $P < 0.0001$), temperature (Wald $\chi^2 = 7.54$, $P = 0.023$), and the interaction between them (Wald $\chi^2 = 9.94$, $P = 0.04$). In general, mortality increased with decreasing salinity. However, the effect of salinity was dependent on the temperature treatments. At both 18 and 35°C, there were significant differences among all three salinities ($P < 0.05$). At 40°C, 5 psu was different than both 10 and 33 psu ($P < 0.0001$), but 10 and 33 psu were not different than each other (Appendix S1: Table S2). At 33 psu, 40°C resulted in more mortality than 35°C ($P = 0.039$), but there were no other differences among temperatures at any salinity level (Appendix S1: Table S2). Importantly, the signs of the interaction parameter estimates gave no indication of a statistically significant synergism (Appendix S1: Table S3). When stressors were decoupled by four weeks, we found significant effects of salinity (Wald $\chi^2 = 234.32$, $P < 0.0001$), but not of temperature (Wald $\chi^2 = 5.00$,

$P = 0.08$) or an interaction (Wald $\chi^2 = 4.38$, $P = 0.36$). Mortality increased with decreasing salinity and all salinities were different from one another ($P < 0.01$; Appendix S1: Table S2).

Discussion

Temporal patterning of multiple stressors

Our results show that the timing of stressors is critical for predicting their effects and interactions. Recovery times of two or four weeks between low salinity and high aerial temperature stress eliminated the synergism that was present when these two stressors were coincident (Fig. 3, Appendix S1: Table S3). This result highlights that different temporal patterns of stressors can completely change their interaction. In this case, a time lag shifted the interaction of two stressors from having a synergistic effect to having no interaction.

The importance of temporal patterns of multiple stressors may become increasingly relevant with climate change. In addition to changing stressor magnitudes and increasing the frequency of extreme events, climate change is altering the timing of stressful events (IPCC 2014). In San Francisco Bay, climate change is projected to increase winter and spring air temperatures and advance spring snow melt (Cloern et al. 2011). These changes might bring the seasonal timing of high temperature and low salinity events closer together, which could result in more coincident events. However, climate change may also result in more dry periods with less runoff from snowmelt and precipitation. In this case, the low salinity season may be short and separation between low salinity and high temperature events may be longer. In either case, the timing of stressors will certainly be affected by climate change and is therefore important to consider when predicting future impacts.

Importance of extreme events

Our results demonstrate that extreme levels of climate-driven stressors can have important implications for population response to climate change and that even modest stress can cause significant impacts, depending on timing. Here, extreme levels of salinity (5 psu) and air temperature (40°C), which mimic major precipitation events or heat waves, resulted in significant oyster mortality, suggesting that testing mean changes in environmental variables provides only a partial picture of organism response to climate change. When paired coincidentally, even stressors that are modest on their own can cause significant oyster mortality when combined. Here, we highlight that extreme events (such as a 40°C heat wave or a 5 psu low salinity event) can be important for predicting ecological dynamics, but also that the co-occurrence of less extreme events (like a 35°C heat wave and a 10 psu low salinity event) can lead to extreme ecological consequences. Increasingly, it is

recognized that extreme ecological events, such as mass mortalities or sudden distributional shifts in intertidal algae (Harley and Paine 2009) can be caused by the co-occurrence of relatively normal stressors, resulting in a compound, multiple-stressor event (Denny et al. 2009).

Physiological mechanisms of stressor effects

It is difficult to predict whether stressors that occur sequentially or decoupled in time will result in synergistic, antagonistic, or additive effects. This difficulty arises because organismal response to multiple stressors depends on the specific stressors under consideration (Helmuth et al. 2005). Two mechanisms that lead to non-additive interactions are cross-susceptibility and cross-tolerance (Todgham and Stillman 2013). Cross-susceptibility arises when exposure to an initial stressor results in decreased tolerance for a second stressor (Chen and Stillman 2012) resulting in a synergistic interaction. For example, a study of an Asian sea cucumber found that increased seawater temperature led to decreased salinity tolerance (Hu et al. 2010). Cross-tolerance occurs when a first stressor increases tolerance for a second stressor (Todgham et al. 2005, Todgham and Stillman 2013) leading to an antagonistic interaction. For example, an initial heat shock at 12°C conferred greater tolerance to osmotic and hypoxic stress in tidepool sculpins (Todgham et al. 2005).

These interactions may be controlled by the degree of similarity between individual stressor effects (Christensen et al. 2006). If two stressors impact organisms through the same mechanism, the initial stressor might prepare the organism for greater tolerance to the second stressor (Christensen et al. 2006, Sinclair et al. 2013). In particular, cross-tolerance is often thought to involve heat shock proteins (Todgham et al. 2005), which are produced in response to many different stressors (Sinclair et al. 2013). On the other hand, stressors that impact different, but related physiological processes might be more likely to produce cross-susceptibility and result in synergistic effects (Breitburg and Riedel 2005).

The occurrence of cross-tolerance or cross-susceptibility can also depend on the stressor magnitudes and temporal patterning (Todgham et al. 2005, Chen and Stillman 2012). Brief time periods between stressors can result in either cross-tolerance or cross-susceptibility (Todgham and Stillman 2013). In this study, Olympia oysters exhibited cross-susceptibility to low salinity and high air temperature when they were applied coincidentally. As we found in this study, long recovery times between stressors have been shown to result in complete decoupling of stressor impacts (Cheng et al. 2015). For Olympia oysters, recovery time of 4 weeks between stressors resulted in stressor decoupling as opposed to cross-susceptibility.

Understanding the physiological mechanisms underlying responses to multiple stressors can help identify general patterns and enable more accurate predictions of impacts from climate-driven multiple stressors. For example, most bivalves do not actively osmoregulate; instead, they respond to stressful salinity by sealing their mantle cavities (Shumway 1977) and switching to anaerobic metabolism (de Zwaan and Wijsman 1976). In general, intertidal bivalves have two options when out of water: remain aerobic via periodic gaping, which is efficient but risks desiccation, or seal their valves and switch to anaerobic metabolism, which prevents desiccation but utilizes additional energy and results in waste accumulation (Ellington 1983, McMahon 1988). Olympia oysters, which are sealed during low tide (J. Bible, *personal observation*), likely go anaerobic during emersion. Following aerial and/or thermal stress, bivalves can be oxygen limited (McMahon 1988, Somero 2002) and upon submergence often open their valves within minutes, re-establishing filter-feeding, bringing oxygen to deprived tissues, and excreting accumulated waste products (Connor and Gracey 2012). Experiencing back-to-back low salinity and high air temperature, as in our coincident treatment, is likely very stressful because both stressors cause anaerobiosis. In particular, if after high air temperature exposure, oysters are submerged in low salinity water, they either open their valves and risk osmotic stress, or stay sealed and cannot re-establish any of the important physiological functions that were halted during emersion. Results from our study suggest that awareness of organismal physiology and response to stress can aid in developing more robust hypotheses about the responses of different taxa to multiple stressors.

Implications for climate change impacts

As environmental stressors increase in magnitude, duration (Miller et al. 2009), and variability (IPCC 2014), juvenile and adult oysters and other sessile or low-mobility organisms that cannot relocate to seek refuge will likely increase the frequency with which they confront their tolerance thresholds. For oysters, exposure to either low salinity or high air temperature alone can pose a risk. However, oysters are exposed to considerably greater threats when they experience both of these stressors coincidentally. The projected increase in variability and changes in temporal patterning of climate stressors will likely change the degree to which stressors overlap, with important consequences for organism survival. Our results highlight that in order to assess the risks associated with climate-related stressors, we need to understand not only how coincident multiple stressors affect organisms, but also how their temporal patterning mediates the impacts of climate change.

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