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Testing local and global stressor impacts on a coastal foundation species using an ecologically realistic framework

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Abstract

Despite the abundance of literature on organismal responses to multiple environmental stressors, most studies have not matched the timing of experimental manipulations with the temporal pattern of stressors in nature. We test the interactive effects of diel-cycling hypoxia with both warming and decreased salinities using ecologically realistic exposures. Surprisingly, we found no evidence of negative synergistic effects on *Olympia* oyster growth; rather, we found only additive and opposing effects of hypoxia (detrimental) and warming (beneficial). We suspect that diel-cycling provided a temporal refuge that allowed physiological compensation. We also tested for latent effects of warming and hypoxia to low-salinity tolerance using a seasonal delay between stressor events. However, we did not find a latent effect, rather a threshold survival response to low salinity that was independent of early life-history exposure to warming or hypoxia. The absence of synergism is likely the result of stressor treatments that mirror the natural timing of environmental stressors. We provide environmental context for laboratory experimental data by examining field time series environmental data from four North American west coast estuaries and find heterogeneous environmental signals that characterize each estuary, suggesting that the potential stressor exposure to oysters will drastically differ over moderate spatial scales. This heterogeneity implies that efforts to conserve and restore oysters will require an adaptive approach that incorporates knowledge of local conditions. We conclude that studies of multiple environmental stressors can be greatly improved by integrating ecologically realistic exposure and timing of stressors found in nature with organismal life-history traits.

Keywords: additive, climate change, diel-cycling hypoxia, latent, multiple stressors, *Olympia* oyster, *Ostrea lurida*, salinity, synergy, warming

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Introduction

Climate change is predicted to have substantial effects on nearly all living organisms planetwide (Walther *et al.*, 2002; Thomas *et al.*, 2004). In addition, local-scale environmental stressors (e.g. land-use change, invasive species, pathogens, contaminants) operate in conjunction with climate change and can have large implications for organismal function and population persistence (Sala *et al.*, 2000; Jetz *et al.*, 2007). Both global and local stressors can interact in nonlinear ways that can produce sudden transitions between alternate

states (e.g. clear vs. eutrophic 'green' lakes; Scheffer *et al.*, 2001; Hobbs *et al.*, 2009), and can frustrate attempts to predict dynamics based on responses to individual stressors. To understand the potential for interacting effects in coastal systems, there has been a proliferation of research investigating the effects of multiple stressors (both global and local in scale) on a range of organisms (e.g. Vinebrooke *et al.*, 2004; Crain *et al.*, 2008; Todgham & Stillman, 2013). While the majority of previous studies have tested simultaneous and constant stress, the temporal patterning of multiple stressors in nature is often not simultaneous and exhibits variation that may critically alter organismal responses.

Developing a generalized theory of organismal responses to multiple stressor impacts has proven

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challenging, in part because of complex variation in the temporal and spatial extent of environmental stressors in nature (Schulte *et al.*, 2011; Niehaus *et al.*, 2012; Pincebourde *et al.*, 2012). An individual stressor may be constant or variable across timescales (e.g. exhibiting diel-cycling, seasonal, or annual variation); however, most stressor experiments utilize constant stressor treatments ('steady state experiments', Davenport, 1982; Schulte *et al.*, 2011), perhaps so that effects can be easily compared across systems and/or because of logistical constraints. In addition, when considering multiple stressors, they may manifest as coincident (simultaneous), sequential, or latent (decoupled in time) stressors. Sequential and latent effects have been well described in response to single stressors (Pechenik, 2006; Hettinger *et al.*, 2012; Scott & Johnston, 2012), but these timescales have not been incorporated into multiple stressor research. The need for 'fluctuating multivariate experimental approaches' has long been recognized (Alderdice, 1963; Davenport, 1982; Wheatly, 1988), but much recent environmental stressor work has largely focused on constant stressor treatments (but see Niehaus *et al.*, 2012; Pincebourde *et al.*, 2012). As such, there is a pressing need to incorporate ecological realism in studies of multiple stressors to accurately assess the impacts of local- and global-scale environmental change (Wernberg *et al.*, 2012).

Multiple stressors may produce additive (linear) effects that are cumulative and reflect the sum of each individual effect alone. Alternatively, multiple stressors can result in synergistic or antagonistic interactions (nonlinear effects) that are greater than or less than the sum of each individual effect, respectively (Crain *et al.*, 2008). One mechanism that may produce nonlinear effects is cross-tolerance, when one stressor increases tolerance to a secondary stressor, potentially resulting in stressor antagonism. Cross-tolerance is thought to increase an organism's ability to mount a cellular stress response to secondary stressors (Todgham *et al.*, 2005). In contrast, nonlinear effects may also arise from cross-susceptibility, when a stressor reduces tolerance to another stressor (Chen & Stillman, 2012), potentially inducing synergistic effects. For example, higher temperature causes an exponential increase in metabolic rates, resulting in increased oxygen demand, which can exacerbate the impacts of hypoxia (oxygen-depleted waters; Vaquer-Sunyer & Duarte, 2008; McBryan *et al.*, 2013).

Among the many important global stressors is the warming of surface waters, which can influence the physiology and ecology of virtually all aquatic organisms (Rosenzweig *et al.*, 2008), and even small increases in temperature can significantly impair physiological function (Pörtner & Knust, 2007). Warming in coastal

ecosystems can occur in tandem with other global-scale stressors (e.g. ocean acidification; Parker *et al.*, 2010; Byrne, 2011; Gazeau *et al.*, 2013) and/or local stressors such as hypoxia (Vaquer-Sunyer & Duarte, 2008). Hypoxia is increasing in spatial extent and intensity in large part due to greater anthropogenic nutrient input (Diaz & Rosenberg, 2008). Furthermore, hypoxia is hypothesized to synergistically interact with warming because warmer waters hold less dissolved oxygen and promote a more stratified water column that limits the diffusion of oxygen into bottom waters (Vaquer-Sunyer & Duarte, 2008; Altieri & Gedan, 2014). Warming also increases the metabolic rate and oxygen demand of organisms and can interact with hypoxia to reduce survival times under oxygen depletion (Vaquer-Sunyer & Duarte, 2011). However, these conclusions are derived from studies that exposed species to constant hypoxia and warming conditions. Diel-cycling hypoxia (daily oscillation of oxygen depletion driven by the balance of photosynthesis and respiration) is commonly observed in near-shore marine ecosystems (Diaz & Rosenberg, 2008), and the temporal variability of this stressor may alter its interaction with temperature, but has not been examined. Hypoxia and warming may also interact with latent environmental stressors, such as low salinity, that are driven by changing patterns of precipitation. In this case, atmospheric warming drives a near exponential increase in atmospheric water-holding capacity, increasing the frequency of extreme precipitation events (Bromirski *et al.*, 2003; Min *et al.*, 2011) that can substantially reduce seawater salinity and increase the osmoregulatory stress experienced by estuarine organisms. However, estuaries in Mediterranean climates that experience distinct wet and dry seasons, such as those along the coasts of California and southern Oregon, are typically exposed to low-salinity events only during the winter wet season and are temporally decoupled from the summer dry season when maximum warming and hypoxia occur.

Global and local stressors are negatively affecting coastal ecosystems and critical species within them such as oysters (Lotze *et al.*, 2006; Waldbusser & Salisbury, 2014). Worldwide, oyster stocks have suffered up to 90% declines in abundance, and as much as 85% of oyster reefs have been entirely eliminated following overfishing and habitat degradation (Beck *et al.*, 2011; Zu Ermgassen *et al.*, 2012). Along the west coast of North America, Olympia oysters (*Ostrea lurida*) are an important foundation species that increase the diversity of associated species by provisioning additional habitat (Kimbrow & Grosholz, 2006). Oysters settle from a larval planktonic stage in the summer when they may be exposed concurrently to diel-cycling hypoxia (Hughes *et al.*, 2011) and thermal stress (Fig. 1). If oysters

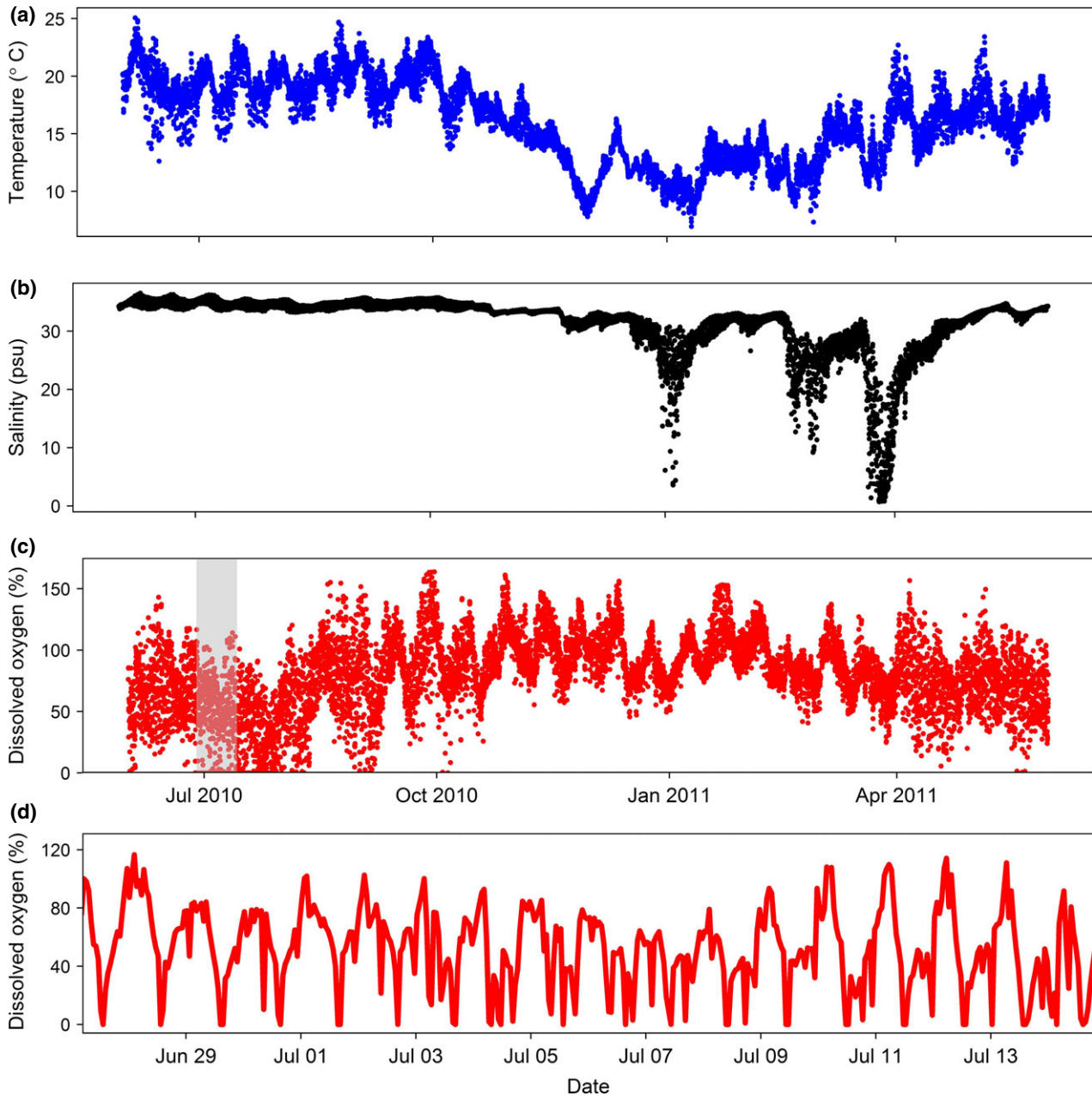


Fig. 1 Annual time series of (a) temperature, (b) salinity, and (c) dissolved oxygen from Kirby Park, Elkhorn Slough, CA, USA, from June 01, 2010 to May 30, 2011. Shaded region in (c) refers to period of coverage for (d) diel-cycling hypoxia from June 28, 2010 to July 14, 2010. Temperature stress and diel-cycling hypoxia occur in the summer months when oysters are recruiting from the plankton. Low-salinity stress presents an osmoregulatory challenge that is offset from temperature and hypoxia, occurring only during winters of Mediterranean climate estuaries. Data are from the Monterey Bay Aquarium Research Institute Land/Ocean Biogeochemical Observatory L02 sensor.

survive these stressors, they then experience benign environmental conditions (i.e. normoxia, decreasing temperature, high salinity) for several months until winter storms deliver precipitation that reduces estuarine salinity and increases osmoregulatory stress (Fig. 1).

Here, we used an ecologically realistic framework to explore multiple stressor impacts upon *Olympia* oysters, a species of conservation and restoration concern (McGraw, 2009). Our primary goal was to experimentally examine the potential interaction of global-scale environmental stressors (warming and low salinity)

with local-scale stress (diel-cycling hypoxia) under a temporal schedule that followed the life-history traits of the Olympia oyster. We quantified stressor effects on oyster survival and growth because they are key components of population demography and organismal fitness. Survival and growth are also easily tracked using image analysis, a nondestructive approach that allows the use of the same individuals across all stressor experiments. Our secondary goal was to compare organismal responses to environmental exposure across North American west coast estuaries (spanning 760 km), to understand the regional-scale environmental context under which oysters undergo stress.

Materials and methods

Stressor levels and experimental design

To test for multiple stressor effects, we used newly settled oysters reared from San Francisco Bay adult broodstock at the Bodega Marine Laboratory (for husbandry details see Data S1). We used this life stage because invertebrate postsettlement juveniles are known to be sensitive to physiological stress and have high mortality, sometimes exceeding mortality rates found during the larval stage (reviewed in Gosselin & Qian, 1997; Hunt & Scheibling, 1997). In addition, settled juveniles are much longer lived than larvae, which allowed us to test exposures to stressors over greater time periods with high replication and statistical power to discern treatment effects. Oysters were settled onto 10 × 10 cm PVC tiles and standardized to 20 oysters per tile. We randomly assigned these experimental oyster tiles to one of six initial stressor treatments comprised of two temperature levels (20 and 24 °C) and three dissolved oxygen levels (extreme hypoxia, hypoxia, normoxia, defined below) in a stratified factorial design (referred to as 'Phase 1'). The 20 °C temperature reflects average water temperatures during the summer in Californian estuaries; 24 °C is a more extreme temperature that is observed only for short periods of time in San Francisco Bay and Elkhorn Slough (Fig. 1). Dissolved oxygen levels were chosen without *a priori* knowledge of specific hypoxia thresholds for this species. Therefore, we selected 2.0 mg L⁻¹ as a moderate level of hypoxia (referred to as 'hypoxia') and 0.6 mg L⁻¹ as a more extreme level of oxygen depletion (referred to as 'extreme hypoxia'). Control dissolved oxygen levels (referred to as 'normoxia') were maintained at 6.4–7.0 mg L⁻¹ (depending on water temperature). Each treatment level had three tank replicates (2 temperature × 3 dissolved oxygen × 3 tank replicates × 12 tile replicates = 216 experimental units). Dissolved oxygen treatments were maintained by covering the seawater surface of all tanks with plastic to limit gas exchange and bubbling the water column with the following: 100% nitrogen gas (extreme hypoxia), 85% nitrogen and 15% ambient air (hypoxia), or 100% ambient air (normoxia). Both hypoxia manipulations were applied for 10 h every night on a diel cycle using electronic actuated valves, simulating diel-cycling hypoxia (Fig. S1) commonly observed in U.S. west

coast estuaries (Fig. 1). After 14 days of the initial experimental treatments, we photographed each tile (Fig. S2) for growth measurements (shell area) using image analysis software (IMAGEJ version 1.46; National Institutes of Health, Bethesda, MD, USA). Shell area is a nonlethal measurement of oyster size and is correlated with dry tissue weight (Hettinger *et al.*, 2012). We measured oyster size (cm²) by randomly selecting half of the oyster images (N = 108) and a random subset of eight oysters per tile. To quantify survival during this phase, we surveyed all tiles for the number of living and dead oysters. An oyster was determined to be dead if it was gaping or unresponsive to probing (oysters typically contract their valves when probed). After this 14-day experimental phase, environmental conditions were returned to normoxia and 20 °C.

To evaluate the persistence of these early life-history stressor effects and simulate latency in salinity stress (Fig. 1), we exposed oysters to benign environmental conditions (typical during the fall season in western U.S. estuaries) for 86 days after the initial temperature and dissolved oxygen treatment ('Phase 2'). Initially, oysters were held at 20 °C for 30 days. After this time, the temperature was decreased by 1 °C per week for an additional 56 days to a target of 12 °C, which follows the average temperature trajectory entering the winter months (Fig. 1). On the last day of Phase 2, we photographed each tile for image analysis as in Phase 1.

To simulate acute low-salinity events typical of the winter season in western U.S. estuaries, we conducted a salinity trial ('Phase 3') using the same 216 oyster tiles from the previous experiments. Each oyster plate was assigned to one of four constant salinity treatments (33.0, 15.0, 10.0, and 5.0 psu) using a stratified random design to ensure equal representation of early life-history treatments (temperature and dissolved oxygen) across salinity treatments. Because the identity of each oyster plate is tracked throughout all experiments, we are able to assess the interaction between early life-history exposure to stress and salinity tolerance. The salinity manipulation was conducted for 8 days because pilot experiments indicated that this duration is the beginning of oyster mortality in response to low-salinity stress (Fig. S4). Pilot experiments also indicated no difference in salinity tolerance between oysters raised from San Francisco Bay and Elkhorn Slough broodstock (Fig. S4), which suggests that these results generalize to both estuaries. Across western U.S. estuaries, low-salinity events (<15.0 psu) are common but rarely last more than 6 days. The 33.0 and 15.0 psu treatments therefore reflect current and commonly experienced levels of salinity, whereas 10.0 and 5.0 psu are more extreme and have only been observed for the north bay region of San Francisco Bay. We chose these latter two values to reflect a continuum of potential extreme events that may be expected under future climate change. The salinity manipulations were carried out in 80-L aquaria with 3 tank replicates per treatment, yielding a total of 12 experimental aquaria. Oysters were acclimated to the target salinity by adding distilled water at a rate of 5.0 psu day⁻¹ and were held at 12 °C for the duration of this experimental phase. After the low-salinity treatment exposure, we increased seawater salinity to 33.5 psu and monitored oysters for an additional 14 days to account for delayed mortality.

Statistics

To measure the effect of environmental stressor treatments on oyster growth, we used linear mixed models (LMMs) with a Gaussian error distribution and identity link function. Oyster size was analyzed at the end of Phase 1 (day 14) and after Phase 2 (day 100). For these analyses, we used the fixed effects of temperature, dissolved oxygen, and their interaction to predict oyster size. For random effects, we used tank and tile nested within tank. We used the Kenward–Roger approximation for denominator degrees of freedom. To meet the normality and equal variance assumptions, we graphically evaluated the data using probability plots and examined model predictions against residuals. Phase 1 data were natural log-transformed because of increasing variation with dissolved oxygen content, whereas Phase 2 data were untransformed. To confirm no random size bias prior to the experiment, we analyzed initial oyster size (day 0) using temperature and dissolved oxygen treatments and their interaction as fixed effects, which revealed no initial difference in oyster size across treatment groups (temperature – $F_{1,12} = 0.371$, $P = 0.553$; dissolved oxygen – $F_{2,12} = 0.396$, $P = 0.681$; interaction – $F_{2,12} = 0.324$, $P = 0.730$).

To measure the effect of environmental stressor treatments on oyster survival (a binary response), we used generalized linear mixed models (GLMMs) with a binomial error distribution and a logit link function. We assessed oyster survival after Phase 1 and Phase 3 of the experiment as separate analyses because Phase 3 involved a new environmental stressor (low salinity). We used full statistical models in Phase 1 (temperature \times dissolved oxygen) and Phase 3 (temperature \times dissolved oxygen \times salinity) with all possible interaction terms because our analyses were restricted to relatively few independent variables. We evaluated model overdispersion by comparing residuals to residual degrees of freedom. Because we did not find overdispersion in either of these GLMMs, we compared treatment effects using Wald chi-squared tests. All analyses and graphics were produced using R (R Core Team 2014) and the packages: 'lme4', 'ggplot2', and 'lmerTest'.

Environmental context

To assess the environmental context of *Olympia* oyster environmental stressor tolerance and to parameterize ecologically relevant treatment levels as well as extreme climate change scenarios, we accessed available physical time series data across multiple estuaries along the west coast of the United States where oysters are known to occur (Table 1). Each record was passed through quality assurance and quality control treatments from each providing agency. We then applied a rate of change filter to the data to exclude abnormal values that reflect instrument noise. For temperature and dissolved oxygen, we truncated data to complete years (see Table 1 for dates of coverage) to eliminate bias from partial annual records and plotted kernel density estimates. For each salinity time series record, we calculated the number of events that continuously exceeded a low-salinity threshold (2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 psu) and calculated the duration of time that each event persisted beneath each threshold.

Results

Early life-history stress

Our experiments revealed strong opposing effects of increased temperature and hypoxia. *Olympia* oysters grown in diel-cycling hypoxic treatments (for 14 days under control temperature of 20 °C) were significantly smaller (up to 61.5%) than those grown under normoxia (Fig. 2; Fig. S2, $F_{2,12} = 110.2$, $P < 0.001$). Oysters grown in temperatures simulating future global warming (24 °C) were significantly larger (22.1%) than those grown at a representative current summer temperature (20 °C; Fig. 2; Fig. S2, $F_{2,12} = 23.39$, $P < 0.001$). We found no evidence for an interaction between temperature and dissolved oxygen (additive effects, Fig. 2, $F_{2,12} = 0.074$, $P = 0.929$). In other words, the effects of temperature were consistently positive across all dissolved oxygen treatments and partially offset negative hypoxia effects. Survival of oysters during this initial phase of the experiment was high (Fig. S3; 91.8%, 1928 survivors of 2110), and no treatment effect upon survival was detected for oxygen (Wald $\chi^2 = 2.130$, $P = 0.3448$), temperature (Wald $\chi^2 = 1.007$, $P = 0.3155$), or their interaction (Wald $\chi^2 = 0.1083$, $P = 0.9473$).

Early life-history stress effects were still evident after 86 days of recovery (i.e. exposure to benign environmental conditions). Hypoxia still exhibited negative effects on growth (oysters were up to 24.8% smaller; Fig. 2, $F_{2,12} = 36.85$, $P < 0.001$), whereas elevated temperature exposure induced positive effects on growth (oysters exposed to warmer water were up to 11.7% larger; Fig. 2, $F_{1,12} = 21.55$, $P < 0.001$). The effect size of both these treatments was reduced over time. There was still no evidence for an interactive effect between temperature and dissolved oxygen after 86 days of recovery (additive effects, Fig. 2, $F_{2,12} = 0.199$, $P = 0.822$).

Latent stress

Our experiment also revealed significant lethal effects of low-salinity exposure, but only at the most extreme level and regardless of exposure to hypoxia or temperature during early life history. In other words, we did not find a latent effect of early life-history temperature or hypoxia stress on performance under low salinity. In this experiment, oysters exhibited threshold responses to low salinity (during an 8-day exposure, see Methods, Fig. S4), where survival was high in all treatments except for the most extreme low salinity of 5.0 psu (Fig. 3, Wald $\chi^2 = 438.1$, $P < 0.001$). We found no evidence for two-way interactions (temperature \times salinity, Wald $\chi^2 = 0.672$, $P = 0.880$; salinity \times oxygen,

Table 1 Environmental time series metadata

Estuary	Site	Latitude/Longitude	Years	Source
Elkhorn Slough, CA	Kirby Park (L02)*	36.8408/−121.7478	2004–2012	MBARI
Elkhorn Slough, CA	Azevedo Pond	36.8457/−121.7538	1995–2013	NERRS
San Francisco, CA	San Pablo Strait	37.9647/−122.4283	1989–2006	USGS
San Francisco, CA	China Camp*	38.0012/−122.4604	2006–2013	NERRS
Tomales Bay, CA	Sacramento Landing*	38.1503/−122.9057	2010–2013	UCD
Tomales Bay, CA	Pelican Point	38.1879/−122.9277	2011–2013	BOON
South Slough, OR	Valino Island*	43.3451/−124.3287	1999–2013	NERRS
South Slough, OR	Charleston Bridge	43.3172/−124.3216	2002–2013	NERRS

Data from all stations were analyzed for low-salinity events; whereas temperature and dissolved oxygen data were taken from sites denoted by (*).

BOON, Bodega Ocean Observing Node (Bodega Marine Laboratory); NERRS, National Estuarine Research Reserve System; USGS, United States Geological Survey; UCD, University of California, Davis.

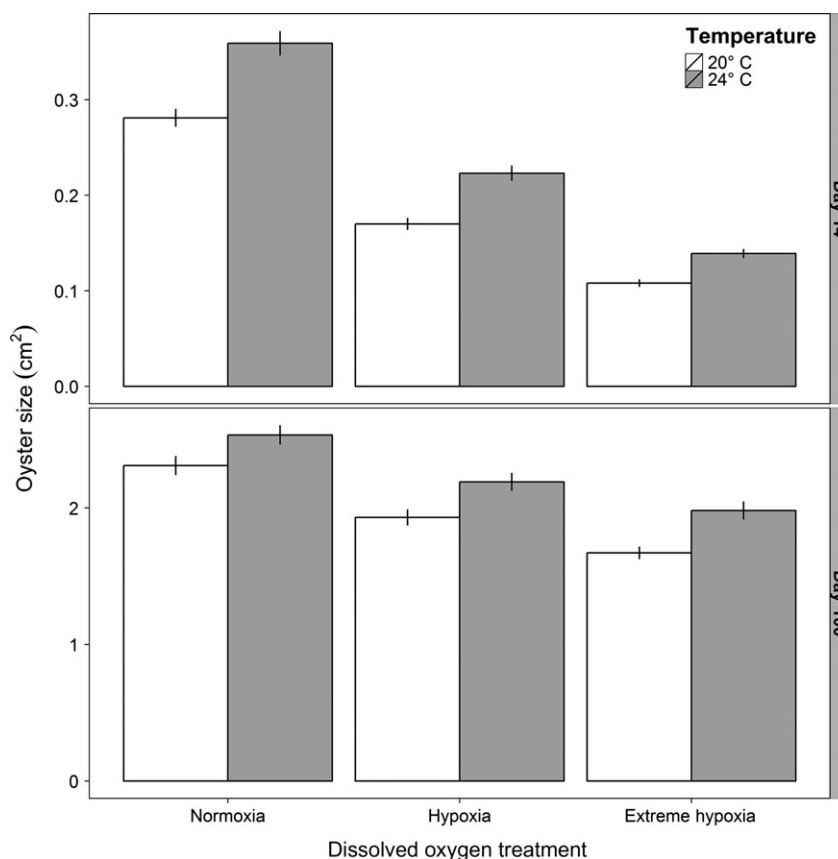


Fig. 2 Oyster size (Mean \pm SEM) following temperature and dissolved oxygen manipulations (normoxia = 6.4–7.0 mg L⁻¹, hypoxia = 2.0 mg L⁻¹, extreme hypoxia = 0.6 mg L⁻¹) immediately after treatments (day 14) and after 86 days of recovery under benign conditions (day 100). Based on model parameter estimates and 95% confidence intervals, all treatment groups are statistically different from each other.

Wald $\chi^2 = 5.81$, $P = 0.444$; temperature \times oxygen, Wald $\chi^2 = 0.483$, $P = 0.785$) or a three-way interaction (salinity \times temperature \times oxygen, Wald $\chi^2 = 2.84$, $P = 0.829$).

Environmental exposure

Our analysis of oyster exposure to environmental conditions in four estuaries along the west coast of North

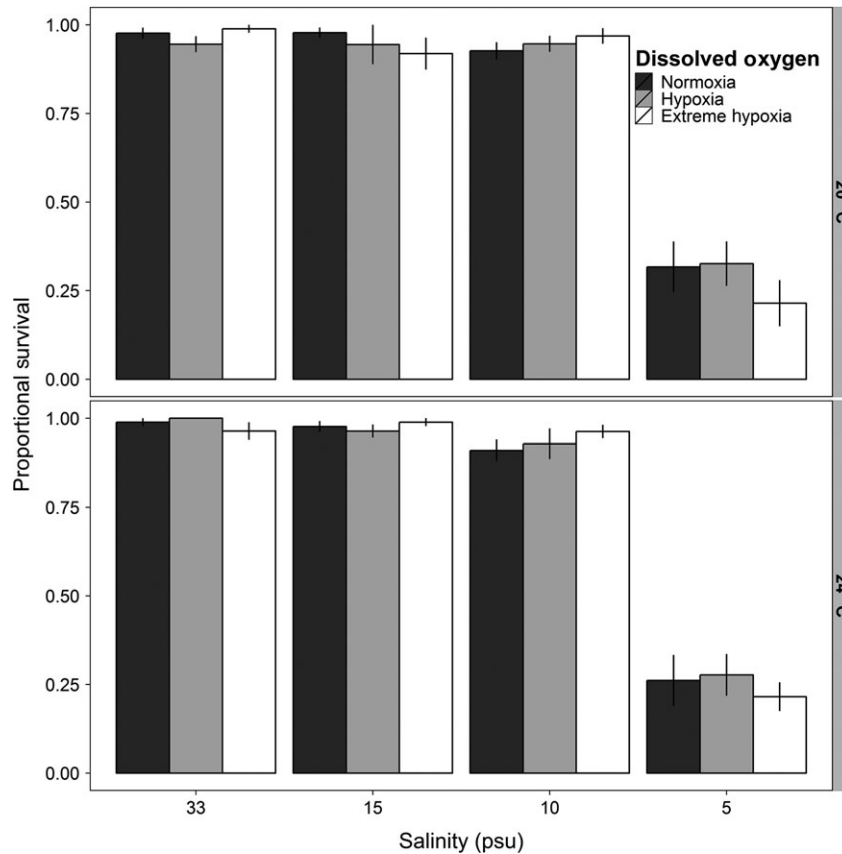


Fig. 3 Proportional oyster survival (Mean \pm SEM) after 8-day exposure to salinity treatments separated by early life-history exposure: dissolved oxygen and temperature treatment. Threshold effects of low salinity on oyster survival appear at 5.0 psu but are unrelated to early life temperature or oxygen treatment.

America (South Slough, Oregon; Tomales Bay, San Francisco Bay, and Elkhorn Slough, California; Table 1) revealed striking differences across estuaries in hypoxia and low-salinity occurrence, but less so for temperature (Fig. 4). San Francisco Bay and Elkhorn Slough were warmest but still infrequently experienced 24 °C (0.2 and 0.3% of observations, respectively; Fig. 4a). Generally, all four sites exhibit broad thermal overlap and suggest high warming tolerance (i.e. difference between LT_{50} and habitat temperature; *sensu* Deutsch *et al.*, 2008; Fig. 4a) especially in Tomales Bay and South Slough. In contrast, dissolved oxygen content was most depressed in Elkhorn Slough and was observed frequently below 2.0 mg L⁻¹ (Fig. 4b). In the summer months (June–August) of 2010 and 2011 at Elkhorn Slough (Kirby Park), 14.0% and 20.1% of all dissolved oxygen measurements were below 2.0 mg L⁻¹. The remaining estuaries all exhibited some degree of hypoxia (<2.0 mg L⁻¹), but these events were typically episodic and not as pronounced as in Elkhorn Slough (Fig. 4b). In terms of low-salinity magnitude and duration, San Francisco Bay exhibited salinities significantly lower

than the other estuaries. According to our lethal salinity experiments, 5.0 psu for 192 h is sufficient to induce oyster mortality (Fig. 3 and Fig. S4). In San Francisco Bay, maximum durations of 111 and 185 h were observed for 5.0 and 7.5 psu, respectively (Fig. 4c). The 7.5 psu treatment is beyond the resolution of our laboratory experiment, and it is possible that this level is sufficient to result in oyster mortality. The remaining estuaries (South Slough, Tomales Bay, and Elkhorn Slough) exhibited low salinities (<5.0 psu), but of durations of <100 h (Fig. 4c) and therefore are not likely extreme enough to induce mortality.

Discussion

The elevation of climate change research to a global research priority has motivated many new studies of multiple stressor impacts (Vinebrooke *et al.*, 2004; Crain *et al.*, 2008; Todgham & Stillman, 2013). This is deservedly so, as the combination of climate change and other anthropogenic stressors may be one of the most pervasive threats to modern day fauna and flora

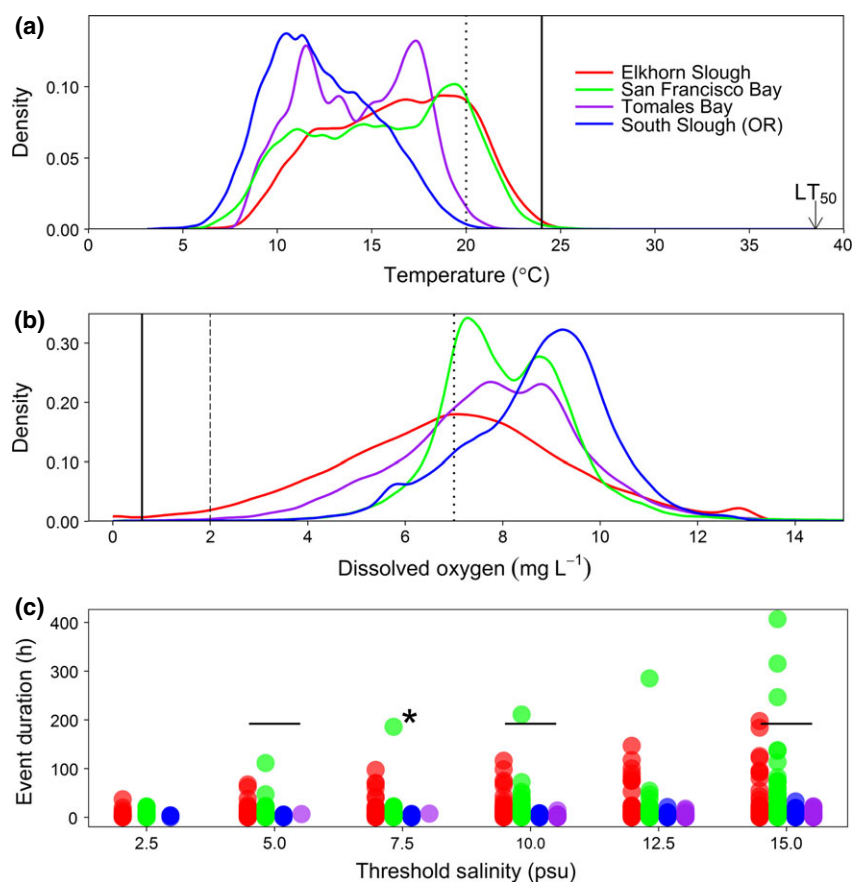


Fig. 4 Environmental conditions across four west coast estuaries at inner bay sites where significant *Olympia* oyster populations are present. (a) Temperature kernel density functions. Dotted line refers to 20 °C control temperature, and solid black line refers to 24 °C warming temperature. Arrow refers to *Olympia* oyster LT_{50} (Brown *et al.*, 2004). (b) Dissolved oxygen kernel density functions. Solid line refers to extreme hypoxia treatment (0.6 mg L⁻¹), dashed line refers to hypoxia treatment (2.0 mg L⁻¹), and dotted line refers to normoxia treatment (7.0 mg L⁻¹). (c) Low-salinity event magnitude and duration. Low-salinity events are calculated as the length of time [event duration (h); *y*-axis] that a continuous salinity time series is below a specified threshold salinity (*x*-axis). Solid bars represent the low-salinity magnitude and duration treatments in our experiment (except control salinity of 33.5 psu). Asterisk refers to low-salinity event that resulted in significant oyster mortality in the field (China Camp, San Francisco Bay, March 2011). See Table 1 for station metadata.

(Sala *et al.*, 2000; Halpern *et al.*, 2008). Recent research incorporating multiple stressors has predominantly focused on chronic coincident stressors, largely revealing nonlinear interactive effects between stressors (i.e. synergism and antagonism). We show that diel-cycling hypoxia and warming generated linear, predictable effects, despite research suggesting that hypoxia and warming are likely to result in synergistic outcomes (Vaquer-Sunyer & Duarte, 2011; McBryan *et al.*, 2013). We suspect that temporal variation in stress (i.e. hypoxia at night and normoxia during the day) mediated the additive vs. synergistic responses. We also found that a high latency between stressors resulted in a decoupling of stressor effects. This suggests that long temporal delays may attenuate latent effects and render multiple stressor effects independent of each other. We

suggest that a more ecologically realistic approach is essential for multiple stressor studies to more accurately assess how different combinations of stressors interact at the organismal level. This approach must also involve a balanced investigation of both local and global stressors across timescales that incorporate the variability and latency found in natural systems.

This study is among the first to test the interaction between warming and diel-cycling hypoxia (but see earlier studies on natural variation in tidepools; Morris & Taylor, 1984; Truchot, 1986). Our results are seemingly divergent from studies and syntheses that have shown synergistic interactions between hypoxia and warming, where impacts of hypoxia are magnified at greater temperatures (Vaquer-Sunyer & Duarte, 2011; McBryan *et al.*, 2013). This difference is most likely due

to the temporal variation in hypoxia that was modeled from field conditions. Prior experiments have almost universally exposed test organisms to chronic hypoxia, which is relevant for systems that experience seasonal or persistent hypoxia such as the Gulf of Mexico or Black Sea (Diaz & Rosenberg, 2008). In contrast, numerous estuarine systems around the world are known to exhibit diel-cycling or periodic hypoxia (Flindt *et al.*, 1997; Diaz & Rosenberg, 2008; Tyler *et al.*, 2009). Therefore, we exposed oysters to a diel-cycling hypoxia regime that simulated daytime normoxia (driven by photosynthesis in nature) as well as nighttime hypoxia (driven by respiration). During hypoxia, oysters undergo metabolic depression, reducing their activity and consumption of oxygen, to better match energy demand with limited energy supply (Widdows *et al.*, 1989). Under warming, ectotherms will exhibit increased metabolic rates and oxygen demand, potentially creating a synergistic interaction with hypoxia. However, warming can also accelerate the acquisition of resources during daytime normoxia, compensating for the oxygen debt incurred during the nighttime and negating synergistic effects if hypoxia is diel cycling (Stickle *et al.*, 1989). Under constant hypoxia exposure, organisms would not have any temporal refuge from low energy supply conditions, thereby responding with synergistic interactions under warming conditions, where energy demand is increased. We caution that this experiment found additive 'linear' effects when measuring growth and survival, metrics that integrate numerous biological processes. However, it is possible that the measurement of other variables (e.g. fecundity) may have yielded synergistic effects.

We suggest that temporal variation and latency between stressors may be a critical determinant in driving linear vs. nonlinear organismal responses. For example, under conditions of high latency, multiple stressors may be additive, whereas low latency may result in nonlinear effects driven by cross-tolerance or cross-susceptibility (Todgham *et al.*, 2005). In this view, our findings of additivity with diel-cycling hypoxia and warming do not conflict with past findings of chronic hypoxia and warming, resulting in synergy. It is also possible that the synergism between warming and hypoxia would only be observed at threshold temperatures that exceed conditions simulated in our experiments (McBryan *et al.*, 2013). Here, the expectation may be that warming partially reduces effects of stressors (e.g. hypoxia) up to a thermal performance optimum. Past the thermal optimum, the interaction may switch to a synergism where warming intensifies other stressors such as hypoxia. Nonetheless, our experimental conditions were based on direct field observations, and in the

near term, these data suggest oysters will first experience additive effects (Fig. 2a).

Positive effects of warming occur when optimal temperatures are greater than habitat temperatures (i.e. positive thermal safety margin; Deutsch *et al.*, 2008), and this suggests a potential for warming to buffer the effects of other environmental stressors. This thermal safety margin has been observed in temperate insects, frogs, lizards, and turtles (Deutsch *et al.*, 2008) and may be due to the more variable thermal environment that temperate latitude organisms experience and subsequent selection for broad thermal tolerance. In contrast, tropical and polar species are selected for narrow thermal tolerance and generally exhibit small thermal safety margins (e.g. Addo-Bediako *et al.*, 2000; Peck *et al.*, 2004; Ghalambor *et al.*, 2006; Pörtner *et al.*, 2007). Several marine examples also support this observation of greater performance at higher temperatures in temperate ectotherms. The intertidal mussel *Mytilus californianus* exhibits greater growth under warmer temperatures in the field (Menge *et al.*, 2008) and the keystone predatory seastar *Pisaster ochraceus*, increased growth rates under warming in laboratory experiments (Gooding *et al.*, 2009). Our experiment subjected *O. lurida* to a temperature (24 °C) never experienced for such a long duration in western U.S. estuaries, and despite this, oysters still exhibited increased growth. Taken together, this suggests that some temperate marine and terrestrial ectotherms possess a significant thermal safety margin and may benefit under limited warming in the near future. It is possible that warming may therefore offset the effects of other global and local stressors for mid-latitude species (Kroeker *et al.*, 2014), whereas tropical species may experience negative effects of warming due to limited acclimation capacity and/or warming tolerance (Deutsch *et al.*, 2008). Despite this apparently large thermal safety margin, we caution that warming may have negative population level consequences that manifest through sublethal or indirect effects such as pathogens or predator-prey interactions (Harvell *et al.*, 2002; Pincebourde *et al.*, 2012).

Our results suggest an important effect of latency, or temporal decoupling, among environmental stressors that can elicit additive rather than synergistic physiological responses to stress. The observed lack of an interaction between the early life stressors (warming and hypoxia) and the latent stressor (low salinity) is likely explained by the large delay in the salinity stress. Here, the latency of low salinity (86-day delay) was likely responsible for unimportant early life-history effects in determining responses to low salinity. Stressor events with decreased latency (less lag time) may be more likely to exhibit synergisms or antagonisms. For example, short latencies (2 weeks) in stressors

(hypoxia and limited resource availability) applied to gastropod larvae have shown latent effects (Li & Chiu, 2013). Greater temporal delays between stressors may thus be more likely to result in additive (and therefore predictable) responses, as we found with *Olympia* oysters. Therefore, the temporal pattern (e.g. variation, coincidence, latency) of climate change stressors with respect to existing local stressors will play a large part in driving biological responses. We suspect that fine-scale temporal variability in stress may be particularly important for sessile species that have limited capacity to behaviorally respond to environmental stress. Mobile organisms may migrate in response to transient stressors (Menge & Sutherland, 1987). In contrast, sessile species such as bivalves must isolate themselves from the ambient environment or mount a biochemical response (Berger & Kharazova, 1997).

It has been proposed that the management of multiple environmental stressors should depend on the nature of the interaction (or lack thereof) among multiple stressors (Brown *et al.*, 2013). Under a scenario involving synergistic interactions, managers should theoretically reduce a local stressor effect because that will mitigate global-scale climate change effects that would otherwise be difficult to reverse. For scenarios with antagonistic interactions, managers should only reduce local stressors within climate refugia, in effect reducing both stressors in tandem. In contrast, additive interactions among multiple stressors dictate that managers should evaluate which stressor effects are greatest and then reduce the stressor with the largest effect (Brown *et al.*, 2013). Here, we show that multiple stressors resulted in additive effects upon *Olympia* oysters, and we suggest that the corresponding management action should consider ameliorating the stressors with the greatest relative effect. However, field data suggest that environmental stress among western U.S. estuaries is highly heterogeneous, and therefore, the management of *Olympia* oysters must be on the local scale. This heterogeneity also suggests the potential for local adaptation in estuarine organisms that have limited population connectivity, which is consistent with recent work indicating that marine populations may be selected for local- or regional-scale environmental conditions (e.g. Pespini *et al.*, 2013). For *Olympia* oysters, low-salinity events that can result in oyster mortality are most likely to be observed in northern San Francisco Bay where freshwater input is greatest due to its large watershed, which drains 40% of the State of California. Indeed, in March of 2011, there was a low-salinity event (<7.5 psu) of nearly 8 days, which resulted in near 100% mortality of oysters at the San Francisco Bay National Estuarine Research Reserve (Fig. 4c and b; B.S. Cheng, unpublished data). However, low-salinity

events of sufficient duration to result in oyster mortality have yet to be measured in Elkhorn Slough, Tomales Bay, and South Slough. In contrast, local stress such as hypoxia is pervasive within Elkhorn Slough (Figs 1 and 4b) where high nutrient inputs and restricted flow structures have created conditions favoring oxygen depletion (Hughes *et al.*, 2011). Hypoxia is expressed to a lesser extent in all of the other estuaries (Fig. 4b), but historically has been a severe issue in south San Francisco Bay (Cloern & Oremland, 1983) and in southern California estuaries that harbor *Olympia* oysters (e.g. Nezlín *et al.*, 2009).

Local actions, such as ameliorating nutrient enrichment or establishing marine protected areas, present a key leverage point for managers to potentially enhance ecosystem resilience under climate change and have been recommended for other eutrophic systems (e.g. Micheli *et al.*, 2012; Falkenberg *et al.*, 2013). Under these multiple stressor scenarios, knowledge of how stressors will interact will be critical for management response. Our ability to predict and therefore prepare for the effects of multiple stressors in natural ecosystems will be enhanced by considering the relative effect of local and global stressors as well as the temporal scale and nature of their interaction as they relate to organismal life history.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Dissolved oxygen conditions for an example 3-day period of the lab experiment.

Figure S2. Experimental tiles after 14 days of exposure to 20 °C and extreme hypoxia (left plate) and 24 °C and normoxia (right plate).

Figure S3. Proportional oyster survival (Mean ± SEM) after the temperature and dissolved oxygen treatments (day 14).

Figure S4. Low salinity duration and oyster survival trial.

Data S1. Experimental oyster broodstock, spawning, and husbandry details.

Table S1. Olympia oyster broodstock collection sites within San Francisco Bay and Elkhorn Slough, California.