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RESEARCH ARTICLE

Multiple-stressor interactions influence embryo development rate in the American horseshoe crab, *Limulus polyphemus*

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ABSTRACT

Fertilized eggs of the American horseshoe crab, *Limulus polyphemus*, are buried in shallow nests above the high tide line, where they are exposed to variations in abiotic conditions during early development. Using a multiple-stressors approach, we examined whether the rate of embryonic development is affected by exposure to combinations of three factors: temperature (25, 30 and 35°C), salinity (5, 15 and 34 ppt) and ambient O_2 (5%, 13% and 21% O_2). Newly fertilized eggs were incubated under 27 fully factorial stressor combinations for 14 days, then allowed to recover in control conditions (30°C, 34 ppt, 21% O_2) for an additional 14 days. Growth rate was measured every 2 days throughout the experiment (*N*=1289). We found that the effect of isolated stressors (high temperature, low salinity or low O_2) reduced developmental success by up to 72% (low salinity), and that stressor combinations showed stronger effects and evidence of complex interactions. For example, low O_2 had little effect individually but was lethal in combination with high temperature, and low temperature in isolation slightly decreased the rate of development but reduced the negative effects of low salinity and low O_2. Development was delayed under exposure to low O_2 but resumed upon return to control conditions after a 10 day lag. These data demonstrate that complex, synergistic interactions among abiotic stressors can substantially alter the development of a coastal invertebrate in ways that may not be predicted from the effects of the stressors in isolation.

KEY WORDS: Multiple abiotic stressors, Compound stressors, Osmotic stress, Hypoxia, Heat stress, Niche space model

INTRODUCTION

Coastal marine environments expose inhabitants to potentially stressful conditions (Denny and Gaines, 2007), the incidence and duration of which are predicted to increase with global climate change, ultimately affecting the inhabiting communities (Harley et al., 2006; Hofmann and Todgham, 2010; Wernberg et al., 2012). Traditionally, studies investigating organism responses to environmental stressors have used a single-stressor approach in a controlled laboratory setting (Wernberg et al., 2012). However, simultaneous exposure to multiple stressors may produce additive, synergistic and/or antagonistic interactions, resulting in physiological responses that are significantly different from responses observed during single-stressor exposure (Crain et al., 2008; Darling and Côté, 2008; Folt et al., 1999). Understanding how multiple stressors interact and affect organisms is essential to predicting the impact of stressors and changing environmental conditions on species survival, biodiversity, community dynamics and ranges (Paine et al., 1998; Russell et al., 2012; Todgham and Stillman, 2013; Tomanek, 2012; Vinebrooke et al., 2004). Among marine invertebrates, this is especially important for early life-history stages, which may be more sensitive to stressors (Byrne and Przeslawski, 2013; Gosselin and Qian, 1997; Pechenik, 1987) – potentially leading to deleterious effects in later developmental stages (Byrne, 2011) – thereby creating a weak link for species' future success (Byrne and Przeslawski, 2013).

The American horseshoe crab, *Limulus polyphemus* (Linneaus), migrates to beaches to nest along the East coast of the USA and the coast of the Gulf of Mexico (Brockmann, 2003; Shuster, 1982). Nesting occurs during biweekly maximum high-tide events (spring tides) along the high-tide line (Brockmann and Johnson, 2011). Females deposit nests of eggs 5–20 cm below the sand surface, where the eggs incubate and develop over the course of 2–4 weeks, hatching in time to emerge from the sand on the following spring tide. Although horseshoe crab embryos are exposed to multiple environmental stressors in the microenvironment below the sand surface where the eggs incubate and develop (Jackson et al., 2008; Penn and Brockmann, 1994; Vasquez et al., 2015), the effects of these stressors on their development is poorly understood. Furthermore, changing global conditions are predicted to increase seawater temperature and precipitation events within the horseshoe crab's geographic distribution (IPCC, 2007), which may exacerbate already stressful conditions (Altieri, 2008). At least some horseshoe crab habitats are already impacted by anthropogenic hypoxia (Diaz and Rosenberg, 2008).

Three characteristics make horseshoe crabs particularly useful for investigating the effects of multiple stressors on development: (1) they are unique among marine invertebrates in nesting on beaches, where they experience a wide range of environmental conditions; (2) during development they are lecithotrophic (non-feeding) until the juvenile stage (Jegla, 1982), simplifying their use in laboratory studies of development; and (3) they are divided into genetically distinct populations across a broad latitudinal range from Maine to Florida, in the Gulf of Mexico, and in the Yucatan Peninsula of Mexico (King et al., 2005), which may have contributed to population-level differences in stressor tolerance.

In this study, we used horseshoe crab embryos from Seahorse Key (SK), FL, USA, to investigate how exposure to 27 fully factorial combinations of temperature (25, 30, 35°C), salinity (5, 15, 34 ppt) and ambient O_2 (5, 13, 21% O_2) for 14 days, followed by a 14 day recovery period, act individually and in combination to affect early development. The maximum and minimum levels of each stressor were designed to be within the maximum and minimum ranges observed in habitats of incubating *L. polyphemus* eggs at SK (Vasquez et al., 2015). Our goal was to identify interactions among

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these stressors and their potential consequences on the survival of developing embryos, and to create a model of the fundamental niche tolerated by developing embryos that is inclusive of their experimental responses to multiple stressors.

RESULTS

Developmental success under control conditions

We defined the control conditions (CC) as 30°C, 34 ppt salinity and normoxia (21% O_2). At SK, microclimate conditions equivalent to CC occur at 5–20 cm below the sand surface at or above the mean high tide line, where the majority of horseshoe crab nests are found (Penn and Brockmann, 1994; Vasquez et al., 2015). Under exposure to CC for 14 days, 75% of fertilized eggs reached a threshold size (TS) of 5 mm^2, indicative of becoming a late-stage embryo (stage 19–21; Shuster and Sekiguchi, 2003), and 81% reached TS by the end of an additional 14 day recovery period at CC (Fig 1; supplementary material Table S3).

We used the Cox proportional hazards model to quantify the hazard ratio (HR) for each treatment in comparison to CC. The HR is a metric traditionally used in medical and biomedical research to assess the 'risk' of a subject succumbing to an illness/death in a given treatment (Spruance et al., 2004). In this study, the HR provides a quantitative method for statistically evaluating whether individuals in a given treatment developed slower, and thus performed more poorly, in a given treatment, compared with individuals reared in CC. Note, however, that HR is used here to represent the 'risk' of developing normally in a given condition divided by the risk of developing normally in CC. Thus, the HR is 1.0 under CC and less than 1.0 for treatments that cause reduced development success.

Single-stressor effects

Horseshoe crab embryos may cease developing under stressful conditions and then resume developing upon return to more permissive conditions (Palumbi and Johnson, 1982). This means that assessment of development solely during stressor exposure may underestimate the embryo's capacity to recover from the stressor and resume development. Therefore, to examine the effect of multiple stressors on developmental success, we exposed fertilized eggs to combinations of temperature, salinity and ambient O_2 for 14 days, followed by a recovery period of 14 days in CC, allowing those embryos that did not develop under exposure conditions the opportunity to develop under more permissive conditions. Throughout the exposure and recovery periods, we assessed developmental success by the proportion of individuals that reached TS (Fig. 2), and these data were then used to compare the HRs for each treatment combination (Fig. 3, with error bars indicating 95% confidence interval, CI). We found a significant difference in developmental responses among the 27 treatment conditions (log-rank test χ^2=717, d.f.=26, P<0.0001). Selected single-stressor treatment effects are highlighted below, with complete results provided in the supplementary material (supplementary material Tables S1–S3).

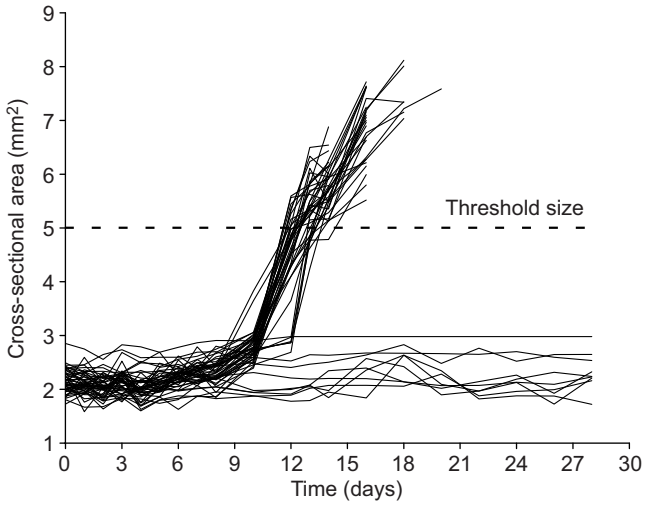

Fig. 1. Growth of developing *Limulus polyphemus* fertilized eggs. Growth under control conditions (N=48; 30°C, 34 ppt, 21% O_2) expressed as cross-sectional area. Cross-sectional area of each individual egg was measured from photographs acquired every other day for 28 days. By day 14, 81% of individuals had reached 5 mm^2 and therefore this threshold size was subsequently used to classify the eggs as developed.

Temperature

The proportion of horseshoe crab eggs that reached TS was significantly affected by temperature alone ($F_{2,1289}$=69.6, P<0.0001) (Table 1). When newly fertilized eggs were exposed to 25°C for 14 days (with salinity and O_2 maintained at CC), TS was reached 4 days later compared with CC (HR: 0.53; 95% CI: 0.34–0.83; Fig. 2A, Fig. 3; supplementary material Table S1), but after a return to 30°C for 14 more days, the proportion reaching TS was 77%, which was not significantly different from CC (supplementary material Tables S2, S3). If newly fertilized eggs were exposed to 35°C for 14 days (with salinity and O_2 maintained at CC), TS was reached 2 days later than in CC (HR: 0.21; 95% CI: 0.12–0.36; Fig. 2A, Fig. 3; supplementary material Table S1), and even after a return to 30°C for 14 days, only 43% of eggs reached TS, which was significantly lower than CC (supplementary material Tables S2, S3).

Salinity

The proportion of fertilized horseshoe crab eggs that reached TS was significantly affected by salinity alone, but only at the lowest salinity of 5 ppt ($F_{2,1289}$=43.1, P<0.0001; Table 1). With the 14 day exposure to 5 ppt salinity (with temperature and O_2 maintained at CC), TS was reached 2 days later than in CC (HR: 0.13; 95% CI: 0.068–0.23; Fig. 2C, Fig. 3; supplementary material Table S1), and even after a return to 34 ppt salinity for 14 days, only 28% of eggs reached TS (supplementary material Tables S2, S3).

Ambient O_2

The proportion of fertilized horseshoe crab eggs that reached TS was significantly affected by ambient O_2 alone ($F_{2,1289}$=8.73, P=0.0002; Table 1). With the 14 day exposure to 13% or 5% O_2 (with temperature and salinity maintained at CC), TS was reached ca. 3 or 12 days later, respectively (13%: HR: 0.49; 95% CI: 0.31–0.77; 5%: HR: 0.3; 95% CI: 0.19–0.47; Fig. 2D,G, Fig. 3; supplementary material Table S1), but after a return to normoxia for 14 days, the proportion reaching TS was not significantly different from CC (supplementary material Tables S2, S3).

Multiple-stressor effects

Selected multiple-stressor treatment effects on the proportion of fertilized horseshoe crab eggs reaching TS are highlighted below, with complete results provided in the supplementary material (supplementary material Tables S1–S3).

Temperature and salinity interaction

There was no significant overall interaction between temperature and salinity ($F_{4,\ 1289}$=1.87, P=0.11; Table 1), but there were

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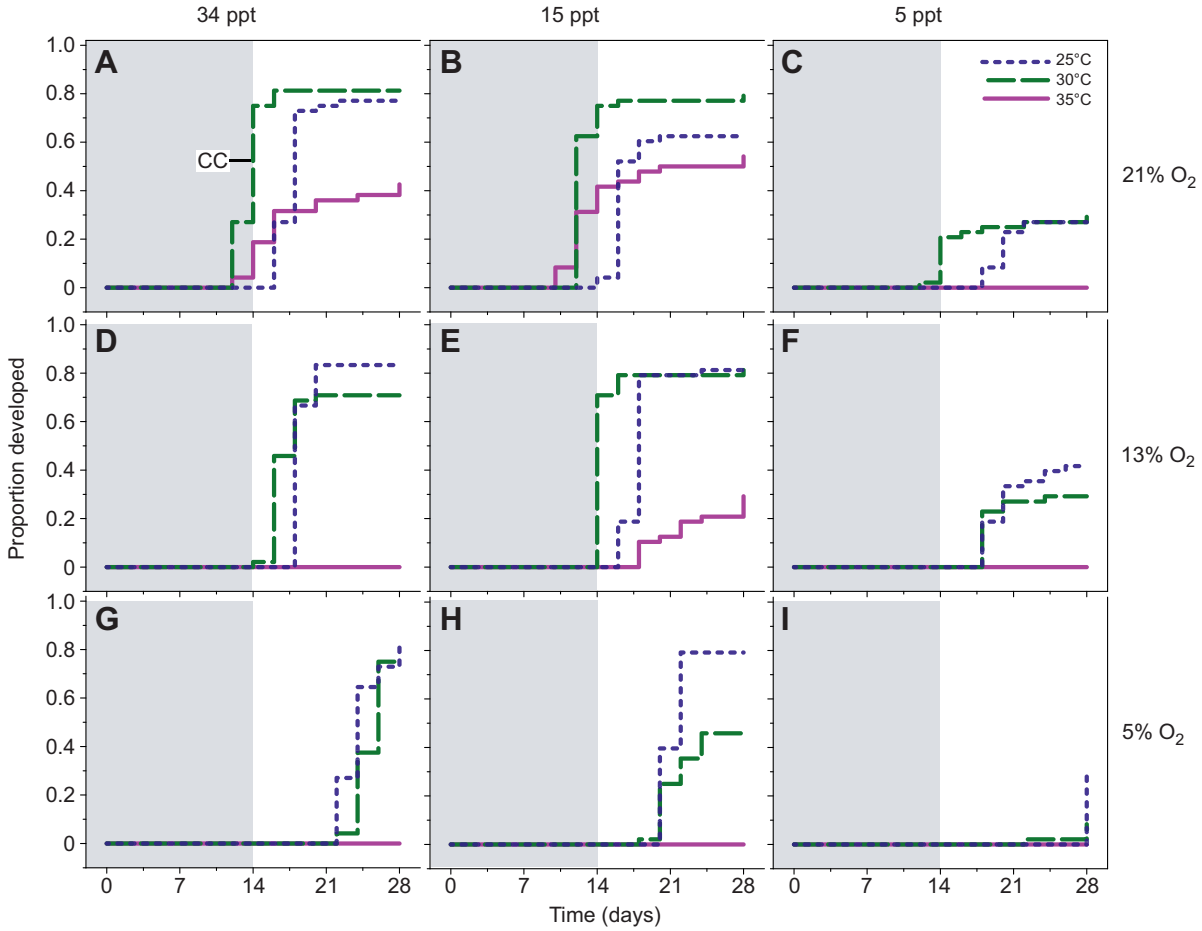

Fig. 2. Kaplan–Meier survival curves for the proportion of *L. polyphemus* eggs developing to threshold size. Survival curves are for eggs from Seahorse Key, FL, USA, that reached the developmental threshold of 5 mm^2 over time for each treatment combination. The temperature conditions of 25, 30 and 35°C are indicated by the colored lines. The following treatment conditions are shown at all three temperatures: (A) 34 ppt salinity and 21% O_2, (B) 15 ppt salinity and 21% O_2, (C) 5 ppt salinity and 21% O_2, (D) 34 ppt salinity and 13% O_2, (E) 15 ppt salinity and 13% O_2, (F) 5 ppt salinity and 13% O_2, (G) 34 ppt salinity and 5% O_2, (H) 15 ppt salinity and 5% O_2, and (I) 5 ppt salinity and 5% O_2. Eggs were exposed for 14 days (shaded half of each panel) followed by 14 days of 'recovery' in control conditions (30°C, 34 ppt salinity, 21% O_2). The survival curve for eggs exposed to control conditions through the treatment and recovery periods is indicated by 'CC' in A. Each line represents 7–8 eggs from each of 6 mating pairs (1289 total eggs).

significant individual treatment differences (supplementary material Table S2). For example, 14 days of exposure to increased temperature combined with moderately decreased salinity (35°C, 15 ppt salinity, 21% O_2) reduced the rate at which TS was reached (HR: 0.33; 95% CI: 0.2–0.54; Fig. 3; supplementary material Table S1), and even after a return to CC for 14 days, the proportion

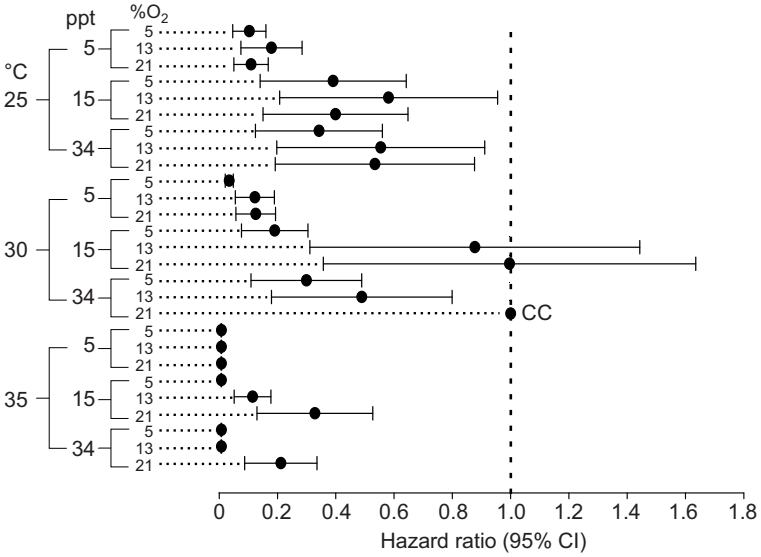

Fig. 3. Hazard ratios for rate of development. Hazard ratios (95% confidence intervals, CI) for the rate at which fertilized *L. polyphemus* eggs from SK reached the developmental threshold of 5 mm^2 for each of the 27 treatment conditions compared with the control treatment (30°C, 34 ppt, 21% O_2). The control condition is indicated by CC. Treatments with 95% CI that cross over the vertical dashed line have rates of development not significantly different from that observed in the control treatment. Hazard ratios less than 1 indicate slower rates of development compared with the control treatment, whereas hazard ratios above 1 indicate faster rates of development compared with the control treatment.

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Table 1. Type III tests of fixed effects

Effect	Numerator d.f.	Denominator d.f.	*F*-value	*P*-value
Temperature	2	1289	69.55	**<0.0001**
Salinity	2	1289	43.09	**<0.0001**
O_2	2	1289	8.73	**0.0002**
Temperature×salinity	4	1289	1.87	0.11
Temperature×O_2	4	1289	7.50	**<0.0001**
Salinity×O_2	4	1289	3.01	**0.018**
Temperature×salinity×O_2	8	1289	1.85	0.064

A generalized linear mixed model and a binary distribution with mating pair set as a random effect was used to test for the significance of each main effect and interaction effect on the proportion of fertilized *Limulus polyphemus* eggs from Seahorse Key, FL, USA, to reach the developmental threshold of 5 mm^2 at the end of the 28 day study. Bold indicates significance.

reaching TS was 52%, which was significantly less than under CC (Fig. 2B compared with A; supplementary material Tables S2, S3). By comparison, 14 days of exposure to the same increased temperature but combined with the lowest salinity (35°C, 5 ppt salinity, 21% O_2) resulted in no eggs reaching TS even after a subsequent 14 days in CC (HR: 0.021; 95% CI: 0.003–0.152; Fig. 2C compared with A; supplementary material Tables S1–S3).

Temperature and O_2 interaction

There was a significant two-way interaction effect between temperature and O_2 ($F_{4,1289}$=7.5, P<0.0001; Table 1). Compared with elevated temperature alone and mild hypoxia alone, exposure for 14 days to the two stressors in combination (35°C, 34 ppt salinity, 13% O_2) resulted in no eggs reaching TS (HR: 0.032; 95% CI: 0.004–0.238; supplementary material Tables S1, S3), and even after a return to CC for 14 days, no eggs reached TS (Fig. 2D compared with A; supplementary material Tables S2, S3).

Salinity and O_2 interaction

There was a significant interaction effect between salinity and O_2 ($F_{4,1289}$=3.0, P=0.02; Table 1). Exposure for 14 days to the lowest salinity in combination with the lowest O_2 (30°C, 5 ppt salinity, 5% O_2) resulted in no eggs reaching TS (HR: 0.039; 95% CI: 0.015–0.099; supplementary material Tables S1, S3), and even after a return to CC for 14 days, the proportion of eggs reaching TS was 8%. For comparison, after exposure to 15 ppt salinity and 13% O_2 (30°C, 15 ppt salinity, 13% O_2) followed by 14 days in CC, the proportion of eggs reaching TS was 81% (Fig. 2E compared with I; supplementary material Tables S2, S3). However, for decreased salinity combined with mild hypoxia (30°C, 15 ppt salinity, 13% O_2) compared with CC (30°C, 34 ppt salinity, 21% O_2), we found no difference in the rate at which TS was reached (HR: 1.0; 95% CI: 0.877–1.36; supplementary material Table S1) and the proportion reaching TS after 28 days (Fig. 2E compared with A; supplementary material Tables S2, S3).

Temperature, salinity and O_2 interaction

The three-way interaction between temperature, salinity and O_2 was not significant ($F_{8,1289}$=1.85, P=0.06; Table 1). The strong interaction between elevated temperature (35°C) and the lowest salinity (5 ppt) was independent of O_2, with no eggs reaching TS even after being returned to CC for 14 days (Fig. 2C,F,I; supplementary material Tables S2, S3).

Change in DNA content during stressor exposure

To determine whether embryos classified above as not having reached TS had ceased developing (i.e. had ceased cell division) or were still developing but with a smaller change in egg size, we assessed the DNA content of developing embryos that were exposed to CC for 24 days, to severely decreased salinity (5 ppt salinity) for 14 days followed by 10 days in CC, or to hypoxia (5% O_2) for 14 days followed by 10 days in CC. We found that the average DNA content in eggs exposed to CC increased from day 3, with a final DNA content of 4.5 µg egg^{-1} on day 24 (Fig. 4). In contrast, eggs exposed to severely decreased salinity for 14 days showed little change in DNA content, including during the 10 day recovery

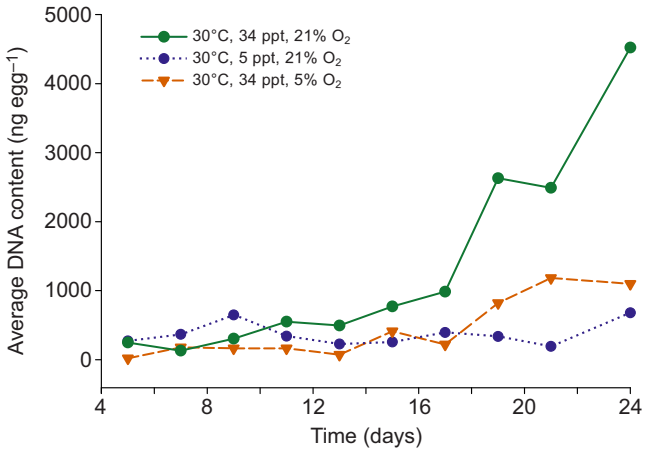

Fig. 4. Average DNA content per *L. polyphemus* egg exposed to three different treatments. Treatment exposure was for the first 14 days under the following conditions: (1) control (30°C, 34 ppt salinity, 21% O_2), (2) severe hyposalinity (30°C, 5 ppt salinity, 21% O_2) and (3) severe hypoxia (30°C, 34 ppt salinity, 5% O_2). On day 14, eggs were transferred to control conditions to recover and continue development.

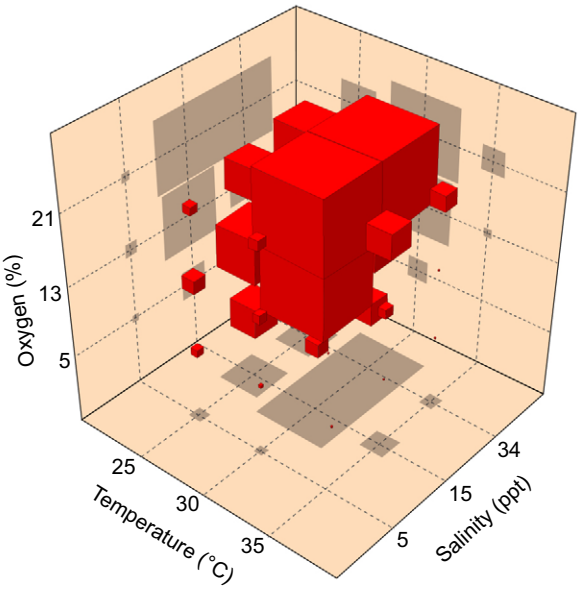

Fig. 5. Hutchinsonian niche-space model depicting the conditions under which developing eggs reached threshold size. The niche-space model plots the hazard ratio for the rate at which fertilized *L. polyphemus* eggs from Seahorse Key reached the developmental threshold of 5 mm^2 for each of the 27 treatment conditions compared with the control treatment of 30°C combined with 34 ppt salinity and 21% O_2. The size of the cube represents the hazard ratio value. Cubes that are similar in size to that of the control treatment indicate similar rates of development. Smaller cubes indicate slower rates of development compared with the rate observed in the control treatment.

period, with a final DNA content of 0.68 µg egg^{-1} on day 24 (Fig. 4). Eggs exposed to hypoxia also showed little increase in DNA content from day 3 to day 14, but upon transfer to CC after day 14, DNA content increased, with a final DNA content of 1.1 µg egg^{-1} on day 24 (Fig. 4).

Hutchinsonian niche-space model of stressor effects

We constructed a Hutchinsonian fundamental niche model (Hutchinson, 1958) for developing horseshoe crab embryos by plotting HR for each of the 27 treatment combinations in 3D space (Fig. 5; supplementary material Movie 1). The size of each cube corresponds to the HR value, and thus is an indicator of development success in comparison to CC at the same time point. Consequently, cubes that are the same size as the CC cube represent similar developmental success, whereas smaller cubes represent conditions at which developmental success is lower. The model demonstrates that under the laboratory conditions used in this study, the fundamental niche of developing horseshoe crab embryos is constrained by elevated temperature and severely decreased salinity. For example, at high temperature and low salinity (35°C, 5 ppt salinity, 21% O_2), no embryos reached TS and therefore no cube is present for that three-condition combination. At low temperature and low salinity (25°C, 5 ppt salinity, 21% O_2), 27% of embryos reached TS (supplementary material Table S3) and thus the cube for that three-condition combination is reduced by 90% compared with the CC cube.

DISCUSSION

Rationale for the multiple-stressor design

Single-stressor studies cannot detect non-additive (synergistic and antagonistic) effects of multiple stressors on organism responses (Crain et al., 2008; Folt et al., 1999), which is critically important because some interactions, such as between elevated temperature and hypoxia, may yield 'ecological surprises' with the potential to cause devastating, species-specific impacts on populations (McBryan et al., 2013; Paine et al., 1998). This may be particularly true for early life-history stages, as they can be more sensitive to stressors (Byrne and Przeslawski, 2013). Wernberg et al. (2012) reviewed published studies from 2000 to 2009 related to marine climate change and found that 65% were single-stressor studies, 30% incorporated two stressors and fewer than 5% included more than two stressors. Recently, the number of two-stressor studies has increased, especially those examining the interactive effects of temperature and ocean acidification (e.g. Harvey et al., 2013; Kroeker et al., 2013), revealing significant interaction effects on marine invertebrate larval survival and development (Byrne and Przeslawski, 2013; Byrne et al., 2013; Hammond and Hofmann, 2012; Hettinger et al., 2013; Padilla-Gamiño et al., 2013).

Logistical complexity is a key reason for the limited number of published studies on multiple-stressor effects in animals. For example, a study that examines just a single response in an organism exposed to three stressors, with each stressor at only three levels (as in the current study), requires assessment of 27 treatment combinations, and this is increased proportionally for each time point. Furthermore, the experimental design is complicated by whether responses will be assessed immediately or following a recovery period, and by the stressor exposure duration (e.g. acute versus chronic) and constancy (e.g. stable versus variable conditions). Moreover, because of the increased degrees of freedom, detecting statistically significant stressor interactions requires more independent replicates than a single-stressor study.

To begin addressing these challenges, we sought a study animal with an early life-history stage that met the following criteria: (1) it is exposed to multiple abiotic stressors in its natural habitat; (2) it can be obtained, maintained and exposed to stressors in the laboratory at high numbers; (3) it would not require feeding during the stressor exposure; and (4) it has a physiologically relevant, fitness-related response to stressors that can be assessed in high numbers at minimal cost. Based on these criteria, we selected horseshoe crab eggs.

Developing horseshoe crab embryos and larvae at SK are exposed to abiotic conditions that are potential stressors, including high and low temperature, low salinity, hypoxia and the presence of hydrogen sulfide, each of which vary seasonally and with tidal height zone and sediment depth (Penn and Brockmann, 1994; Vasquez et al., 2015). In this study, we examined the effects of exposure to temperatures, salinities and ambient O_2 percentages over ranges that are consistent with those observed in the natural habitat. To fully model the environmental conditions at SK, we would have needed to vary diel temperature, circatidal O_2 and salinity over a range to include high to no rainfall, and each of these conditions would need to differ based on the tidal height and sediment depth being modeled. Such an experimental design would have been well beyond the scope of this study, which was intended as an early step in characterizing and understanding how the early life-history stages can be affected by exposure to multiple stressors.

Consequently, we exposed the horseshoe crab eggs to constant stressor conditions that were based on environmentally realistic ranges. For example, microclimate conditions equivalent to the reduced temperature treatment occur at all tidal heights, but are more common deeper in the sand (e.g. 20 cm below the sand surface; Vasquez et al., 2015), and seawater salinity and temperature measured at a monitoring station near SK (6 km northwest at the Gulf Jackson lease area) ranged from 13 to 36 ppt and 7 to 34°C during the 2012 spawning seasons (University of Florida Shellfish Extension, 2012). Temperatures of 35°C and above occur in shallow sediment depths at SK, although these events are rare (Vasquez et al., 2015). Finally, we used an exposure duration of 14 days to represent the time between fertilization and the following spring tide, by which time embryos in the field have typically emerged (Penn and Brockmann, 1994). These stressors are not unique to SK; in at least one other habitat of this species, the Chesapeake Bay, seasonal hypoxia has occurred for the past 50 years (Kemp et al., 2005), seawater salinity varies seasonally with river discharge (Schubel and Pritchard, 1986; Xu et al., 2012), and water temperature is predicted to increase 2–6°C by the end of the 21st century (Najjar et al., 2010).

Significance of stressor effects on fitness

We found that exposure of newly fertilized horseshoe crab eggs to temperature, salinity or hypoxia stress in isolation significantly reduced the proportion of eggs that developed to late-stage embryos by the end of the 28 day study period. Under CC in the laboratory, which is representative of the microenvironment at SK where the majority of horseshoe crabs nest (Penn and Brockmann, 1994; Vasquez et al., 2015), 81% of the fertilized eggs reached TS (i.e. an advanced embryonic stage) within 12 days. In comparison to CC, exposure to certain stressors, especially in combination, reduced the number of embryos that reached TS by the end of the exposure and subsequent recovery period, with some combinations producing significant two-way interactions. We did not detect any significant three-way interactions, which we suspect is due to low statistical power rather than the absence of physiological effects, as three-treatment effects result in high degrees of freedom.

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Developing horseshoe crab eggs depend on agitation and hypo-osmotic shock from tidal inundations to facilitate hatching into a trilobite larva and emergence from the sand (Ehlinger and Tankersley, 2003), and such inundations reach the high tidal height zone located above the mean high tide line only during the biweekly spring tide events. Therefore, horseshoe crabs that experience delayed development may not be competent to emerge from the sand during the following high tide, which is likely to decrease fitness, as remaining in the sand longer increases the risk of predation by shorebirds and other predators (Botton, 2009; Shuster and Sekiguchi, 2003). Therefore, the overall results of our study are consistent with the hypothesis that horseshoe crab mating pairs nest in locations along the beach that maximize successful embryo development (Penn and Brockmann, 1994; Vasquez et al., 2015). However, it must also be noted that there are instances in which delayed development may be an adaptive response, as horseshoe crab eggs that experience long-term exposure to reduced temperatures can overwinter and emerge as trilobite larvae the following spring season (Botton et al., 1992).

Thermal stress

The optimal temperature for larval development in *L. polyphemus* is 25–30°C (Jegla and Costlow, 1982; Laughlin, 1983), with temperatures above 35°C being potentially lethal (Ehlinger and Tankersley, 2004). We found that exposure to 35°C under control salinity and O_2 conditions (34 ppt, 21% O_2) reduced the proportion reaching TS by 38% compared with that at 30°C at the end of the study period. Horseshoe crab embryos do not show an inducible heat shock response (HSR) following a 3 h heat shock at 35°C, 20 ppt and ambient O_2, and may instead maintain high levels of constitutive heat shock proteins (Botton et al., 2006). Therefore, the reduced developmental success we observed at 35°C suggests that the negative effects of heat stress could not be alleviated by a constitutive or inducible HSR.

Salinity stress

The optimal salinity for horseshoe crab development is 20–30 ppt (Jegla and Costlow, 1982; Laughlin, 1983). We found no difference between 15 ppt and 34 ppt salinity in the proportion of embryos that reached TS, but we found a significant reduction (by 53%) in the proportion that reached TS at 5 ppt salinity. We also observed that cell division (as assessed by total DNA content) was reduced during exposure to 5 ppt salinity, and furthermore that this persisted during the subsequent exposure to CC, suggesting that the embryos had died. This is consistent with *L. polyphemus* being unaffected during early development by extended exposure to the typical range of environmental salinities (Vasquez et al., 2015), while being intolerant of extended duration exposure to very low salinity.

Hypoxia stress

At SK, developing embryos may be exposed to hypoxia in deeper sediment depths of the low tidal height zone below the mean high tide line (Vasquez et al., 2015). We observed a developmental delay of ca. 3 or 12 days when embryos were exposed to 13% or 5% O_2, respectively. Our analysis of total DNA content over time indicated that cell division ceased (or was at least severely reduced) throughout hypoxia exposure, but resumed soon after the eggs were transitioned to CC, suggesting metabolic rate depression (Guppy and Withers, 1999). This is consistent with previous studies showing that late-stage horseshoe crab embryos become inactive immediately upon exposure to hypoxia (2.5% O_2), but can be maintained in hypoxia for 9 days and then returned to normoxic conditions, at which point they resume activity (Palumbi and Johnson, 1982). Hemocyanin would be expected to reduce the physiological impact of hypoxia, but it is not present in horseshoe crabs until stage 18 (Coursey et al., 2003). We did not assess the time at which the embryos reached stage 18, but under CC the embryos reached stage 21 at about day 12–14, and this was extended even later under hypoxic conditions. Therefore, most embryos in this study were unlikely to have had sufficient hemocyanin during most or all of the 2 week exposure period to compensate for the reduced availability of O_2 during the hypoxia exposures.

The costs of stress responses

Stressor exposure produces trade-offs in the allocation of energy to survival, stress tolerance and other fitness-related functions, including processes such as the HSR (Feder and Hofmann, 1999; Hershko and Ciechanover, 1998; Lesser, 2006). Consequently, an organism's capacity to tolerate stress exposure may be limited by its available energy and metabolic capacity, which is described by the oxygen-limited and capacity-limited thermal tolerance (OCLTT) concept (Pörtner, 2012, 2010; Sokolova, 2013; Sokolova et al., 2012). For the lecithotrophic horseshoe crab larvae (Jegla, 1982), a set amount of energy (i.e. lipids) is available for growth and homeostatic regulation. Exposure to multiple stressors during development and the resulting increased energy demands necessary to maintain cellular integrity (Sokolova et al., 2012) may also produce latent effects by reducing the energy available for larval settlement and early juvenile growth, and latent effects have been observed for organisms experiencing delayed metamorphosis, food limitation/nutritional stress, salinity stress, increased energy use and pollution (Pechenik, 2006).

Temperature and hypoxia interactions

Thermal stress and hypoxia are common during summer months in the Gulf of Mexico, where large hypoxic 'dead zones' appear as a result of eutrophication (Diaz and Rosenberg, 2008). In a meta-analysis of marine benthic organisms across three taxonomic groups (mollusca, fishes and crustaceans), Vaquer-Sunyer and Duarte (2011) reported that survival time is reduced on average by 74% under exposure to elevated temperature combined with hypoxia, and that there is a significant increase in median lethal O_2 concentration with increasing temperature. At SK during the autumn 2010 mating season, sediment temperatures exceeded 30°C for 25 h in a 2 week period, and the ambient O_2 was as low as 5.9% (Vasquez et al., 2015). We found that the combination of elevated temperature with hypoxia resulted in a synergistic reduction in the proportion of eggs that reached TS, whereas the interaction between decreased temperature (25°C) and hypoxia was antagonistic, both of which are findings consistent with the OCLTT concept.

Salinity and hypoxia interactions

It is unclear whether horseshoe crab eggs would be exposed to hyposalinity and hypoxia in combination under natural conditions. Freshwater influx from precipitation may increase aeration, raising the ambient O_2, but this effect may be reduced or absent deep within the sand, especially in the low tidal height zone where hypoxia and hydrogen sulfide are present (Vasquez et al., 2015). Horseshoe crab embryos are osmoconformers, becoming isosmotic after 6 h of hyposalinity exposure (Ehlinger and Tankersley, 2004), whereas adult horseshoe crabs are osmoconformers above 23 ppt and partial osmoregulators below that salinity (Robertson, 1970).

Even osmoconforming is not a completely passive process; at a minimum, initial active osmoregulation must occur to adjust cellular

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osmolality in order to combat swelling and cell rupture (Charmantier et al., 2009). Cell volume regulation in adult horseshoe crabs, as investigated in isolated heart tissue transferred from 930 to 400 mosmol l^{-1} seawater, occurs initially via efflux of Na^+ and Cl^- ions within the first 24 h of exposure. Ion loss is then followed by efflux of the organic osmolyte glycine betaine, with concentrations decreasing from 48 h to 7 days after osmotic stress exposure. Furthermore, there is overlap between the hyposalinity response and the HSR, as horseshoe crab embryos reared in 20 ppt salinity increase expression of heat shock protein (HSP)70 and HSP90 following a 4 h osmotic shock at 10 ppt salinity (Greene et al., 2011).

As noted above, exposure to 5 ppt salinity caused a reduction of more than 50% in the proportion of fertilized eggs that reached TS. After transition from 5 ppt to CC (which is 34 ppt salinity), many individual embryos were able to recover and resume development, but only if the initial low salinity exposure occurred in combination with normoxia and a temperature of 25–30°C. That is, recovery was not observed after exposure to 5 ppt salinity in combination with thermal stress or severe hypoxia stress. This suggests that the recovery was dependent on the embryo's bio-energetic status, in particular its ability to maintain aerobic metabolism during the initial hyposalinity exposure, as predicted by the OCLTT concept. Consistent with this, O_2 consumption of horseshoe crab trilobite larvae is greater during exposure to 10 ppt salinity than to 25 ppt salinity (Laughlin, 1983). Furthermore, rearing horseshoe crab larvae to the trilobite stage under 10 ppt salinity rather than under 20–35 ppt salinity causes a 25% decrease in ash-free dry mass and an increased time to chorion shedding, fourth embryonic molt and hatching, all of which are consistent with increased energy loss (Greene et al., 2011; Jegla and Costlow, 1982; Laughlin, 1983).

In contrast to the negative synergistic interaction of hyposalinity with severe hypoxia, hyposalinity exposure in combination with mild hypoxia (13% O_2) produced an antagonistic interaction that increased the proportion of embryos reaching TS. One potential explanation would be increased O_2 affinity of hemocyanin due to the decreased chloride ion concentration (e.g. hyposalinity conditions) compared with the chloride ion concentration found in normal seawater (Sullivan et al., 1974; Towle and Henry, 2003). This would increase O_2 delivery under simultaneous exposure to hypoxia, thereby promoting maintenance of cellular responses to salinity stress. However, as noted above, significant hemocyanin is not present in horseshoe crabs until later developmental stages (Coursey et al., 2003).

Hutchinsonian niche-space model of stressor interactions

The Hutchinsonian fundamental niche is an *n*-dimensional hypervolume, where *n* represents the coordinates of the environmental variables within which a species can survive (Hutchinson, 1958). Construction of niche-space models has been applied to predicting how global climate change and increasing sea surface temperatures will influence the spatial distribution of the marine copepod *Calanus finmarchicus* (Beaugrand et al., 2013), and the fundamental niche has been described for the water flea *Daphnia magna* along a pH and calcium gradient (Hooper et al., 2008; Materna et al., 2012). In these examples, the constructed fundamental niche was used to predict the distribution of the organism in natural habitats.

To allow visualization of potential stressor interactions that constrain the niche space, we used the responses to multiple stressors to create a Hutchinsonian niche-space model of developing *L. polyphemus* embryos. While our model is based on exposure durations that are unlikely to occur under natural conditions, it nonetheless demonstrates the feasibility of using multiple-stressor responses to create a multidimensional niche-space model. The visualization emphasizes that embryo development may be especially compromised by the combination of high temperature and precipitation. Summer is when the fewest nesting horseshoe crabs are present (Brockmann and Johnson, 2011; Rudloe, 1980), and horseshoe crab eggs are typically placed at depths in the sand at which the exposure to heat and the effects of precipitation are likely reduced (Vasquez et al., 2015). However, at such sediment depths the eggs are at greater risk of exposure to hypoxia and hydrogen sulfide, and thus the sediment depth at which the female places her eggs may represent a trade-off selected to optimize embryo development (Penn and Brockmann, 1994; Vasquez et al., 2015).

Conclusions

Studies that integrate a multiple-stressors approach will further our understanding of the ecological physiology of organisms by more closely simulating the multivariate environment experienced by marine organisms. This study highlights the complex non-additive interactions experienced by marine invertebrates when exposed to two or more simultaneous stressors that can only be identified in multi-stressor studies. We found that developing horseshoe crab embryos from SK are negatively influenced by elevated temperature, reduced salinity and hypoxia, and that in combination these individual-stressor effects can become synergistic, substantially reducing developmental success. However, in some instances, horseshoe crab embryos are able to recover from the initial stressor exposure and successfully develop under recovery conditions, highlighting the resilience and physiological tolerance of this 'living fossil'.

MATERIALS AND METHODS

Study site and specimen collection

Our research was conducted under a special use permit from the Lower Suwannee National Wildlife Refuge (J. Kasbohm, refuge manager), and a special activity license from the Florida Fish and Wildlife Conservation Commission.

Eggs of the American horseshoe crab, *L. polyphemus*, were collected as previously described (Vasquez et al., 2015) at SK (29°5′47″N, 83°3′55″W), an island located 4 km from Cedar Key, FL, USA, on the northern Gulf Coast of Florida. Horseshoe crabs nest at SK from August to October and March to May each year (Brockmann and Johnson, 2011; Rudloe, 1980). Newly fertilized horseshoe crab eggs were collected from six monandrous horseshoe crab mating pairs on 23 February 2012 during the afternoon high tide. Eggs were placed into plastic dishes containing seawater (31 ppt salinity during collection) for transportation back to the University of Florida laboratory.

Multiple-stressor exposure

Once at the laboratory, 16 eggs from each of the six mating pairs were placed individually into the wells of a 96-multiwell plate prefilled with 200 µl filtered seawater (FSW). A total of 27 multiwell plates were prepared, with one plate per experimental condition. The treatment conditions consisted of 27 fully factorial combinations of salinity (5, 15 and 34 ppt salinity, the last of which is full-strength seawater), ambient O_2 (5, 13 and 21% O_2) and temperature (25, 30 and 35°C). Experimental treatments were based on environmental conditions observed within the beach at SK where horseshoe crab eggs are deposited (Vasquez et al., 2015). The complete study included 432 eggs from each of the six mating pairs, for a total of 2592 eggs.

An automated liquid handling robot (Biomek 2000, Beckman Coulter, Inc., Indianapolis, IN, USA) was used to adjust the salinity in each well of each multiwell plate from the initial salinity of 31 ppt using a gradual process to minimize osmotic shock. For those wells with low salinity, half of the well volume (100 µl) was removed from each well and replaced with

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100 µl deionized water. This was repeated every 30 min until the final treatment condition was obtained. For wells with 34 ppt salinity, the entire volume of 200 µl 31 ppt FSW was removed and replaced with 200 µl 34 ppt FSW. Stock solution salinities were verified by refractometer.

Upon establishing the final salinity, the multiwell plates requiring hypoxic conditions were placed into custom-made gas-tight chambers (Ortega et al., 2008), which were then filled with a combination of O_2 and N_2 gas to achieve 5% or 13% O_2, as appropriate. Multiwell plates assigned to 21% O_2 were not placed into gas-tight chambers but instead were sealed with Parafilm to limit evaporation. The O_2 compositions were verified using a fiber-optic O_2 probe (FOXY-R O_2 probe with NeoFox spectrometer, Ocean Optics, Dunedin, FL, USA), calibrated using room air (21% O_2, normoxia) and 100% N_2 gas (0% O_2). Each chamber or 21% O_2 multiwell plate was then assigned to one of three temperature-controlled incubators for the experimental exposure period of 14 days. Previous studies have reared horseshoe crab embryos on moist paper towels (Ehlinger and Tankersley, 2003) and in multiwell plates (Ehlinger and Tankersley, 2004). In the natural environment, horseshoe embryos and larvae are likely exposed to both moist and submerged conditions, depending on the tidal height and the nest's position on the beach and its depth in the sediment (Penn and Brockmann, 1994). In our study, maintaining the developing horseshoe crabs submerged in multiwell plates allowed for high-throughput imaging.

After the 14 day exposure period, the multiwell plates were removed from the chambers and placed into recovery conditions (30°C, 34 ppt salinity, 21% O_2) for an additional 14 days, to allow development under optimal environmental conditions (Palumbi and Johnson, 1982). Establishing recovery conditions required increasing the seawater salinity from either 5 or 15 ppt salinity to 34 ppt salinity, which was done by removing 100 µl of solution from each well and replacing it with 100 µl of either 15 or 34 ppt FSW over a 4 h time period, as described above.

Throughout the exposure and recovery periods, the chambers were opened every 2 days to allow the conditions to be re-established by exchanging half of the medium in each well with fresh medium at the treatment salinity and re-establishing the O_2 treatment. While the chambers and plates were open, photographs of each well were taken by placing each multiwell plate on an inverted microscope (Olympus IX-70) with a motorized stage (Prior Proscan, Prior Scientific, Rockland, MA, USA) controlled by software (Stage Pro and Scope Pro, Media Cybernetics, Rockville, MD, USA). Images were acquired with a cooled-CCD camera (Retiga 2000R, QImaging, Surrey, BC, Canada). The multiwell plates were then returned to the incubators. This process was completed within 30 min. To track developmental progress, images were obtained every other day for every individual embryo throughout the 28 day study.

The images were subsequently analyzed for cross-sectional area (mm^2) using NIH ImageJ (Rasband, 2012). As noted by Sekiguchi et al. (1982), egg diameter remains relatively constant from stage 1 through to stage 18, which occurred under control conditions in our study on approximately day 10. The transition from the second embryonic molt (stage 19) to a late-stage embryo (stage 20) is characterized by rapid swelling (Sekiguchi et al., 1982). We found that this was clearly evident in the images between days 10 and 14 as an increase in egg cross-sectional area (Fig. 1). All of the eggs that had exceeded 5 mm^2 cross-sectional area were identified as having reached stage 20, whereas those eggs that never swelled ultimately failed to develop (data not shown). After the 14 day treatment exposure, late-stage embryos were active within their embryonic membrane and fully formed with no visible abnormalities. As cross-sectional area was both easily measured and less subjective than identifying developmental stage, we subsequently used a TS of 5 mm^2 to indicate that development had reached or exceeded a late-stage embryo. Data were obtained for half of the embryos (1296 individual horseshoe crab embryos, representing eight embryos from each of the six mating pairs). Technical errors resulted in the loss of seven eggs. Each egg was analyzed at 15 time points, for a total of 19,335 samples.

DNA content

DNA content of developing embryos was analyzed to evaluate developmental progress during stressor exposure. Newly fertilized horseshoe crab eggs were collected on 16 September 2012 during the afternoon high tide at SK from a single monandrous mating pair. Eggs were transported to the laboratory and exposed to one of three treatments for 14 days: control (30°C, 34 ppt salinity, 21% O_2), hypoxia (30°C, 34 ppt salinity, 5% O_2) and hyposalinity (30°C, 5 ppt salinity, 21% O_2). Treatment conditions were established following the same protocol used for multiple-stressor exposure outlined above. After 14 days of exposure, eggs from each treatment were placed into recovery conditions (30°C, 34 ppt salinity, 21% O_2) for an additional 10 days. Conditions were maintained every 2 days throughout the exposure and recovery periods, as described above. Eight eggs were frozen in liquid nitrogen every 2 days beginning on day 5.

For each time point, 1–2 frozen eggs were weighed (5–10 mg) and DNA was extracted and purified into DNA homogenates using the Gentra Puregene Tissue Kit (Qiagen, Valencia, CA, USA). A standard curve was made using calf thymus DNA (D3664, Sigma-Aldrich, Inc., St Louis, MO, USA). DNA homogenates were serially diluted in TNE buffer (10 mmol l^{-1} Tris, pH 7.4, 0.2 mol l^{-1} NaCl, 1 mmol l^{-1} EDTA) to fall within the standard curve range. Diluted DNA homogenates were incubated in the dark with 0.1 µg ml^{-1} Hoechst 33258 dye (Sigma-Aldrich, Inc.) for 10 min, and DNA content was measured in 384-well microplates using a multimode microplate reader (BioTek Synergy™ HT, Winooski, VT, USA) in fluorescence mode at 365 nm excitation and 460 nm emission (sensitivity at 100). Samples were prepared in triplicate. DNA content is expressed as mean DNA (ng) per egg at each time point.

Statistical analysis

A generalized linear mixed model (PROC GLIMMIX; SAS 9.2, SAS Institute Inc., Cary, NC, USA) with a binary distribution, logit link function and Kenward–Roger denominator degrees of freedom for fixed effects method was used to identify significant main effects and significant combinations of effects on the proportion of fertilized eggs that reached TS after 28 days for each treatment. Temperature, salinity and O_2 were set as fixed effects and mating pair was a random effect. A Tukey *post hoc* multiple comparisons test was used to identify significant differences in the proportion of fertilized eggs that reached TS between treatments.

Survival analysis (PROC LIFETEST and PROC PHREG procedures in SAS 9.2) was used to identify differences in the proportion of embryos that had reached TS for each treatment combination, where the day each individual reached TS was classified as an event. Individuals that had not reached TS by the end of the study were censored on day 28. Individuals that were 'lost' during the study (due to accidental aspiration by the liquid handling robot) were censored on the day of their last measurement. Kaplan–Meier (KM) failure curves (using PROC LIFETEST) were created to plot the number of events that occurred over time (28 days) (supplementary material Table S3). Significant differences between KM curves for each treatment were determined using the log-rank test and Šidák *post hoc* analysis for multiple comparisons.

The Cox Proportional Hazards model (using PROC PHREG) with the Efron ties handling method was used to quantify HR for each treatment in comparison to CC. The Cox Proportional Hazards model assumes that the hazards function does not change at any time during the study. The proportional hazards assumption is often evaluated by plotting KM survival curves and identifying whether these curves intersect, which would denote that the proportional hazards assumption is not met, and therefore that the hazard function varies with time for that treatment. However, as there were 27 treatments used within this study, there is a high probability that the KM survival curves would cross, and when tested, the proportional hazards assumption for these data was not met. The traditional Cox Proportional Hazards model was retained, with the proviso that the proportional hazards assumption was violated for some treatment combinations.

In certain treatment combinations that were presumably highly stressful (e.g. 35°C combined with 5 ppt salinity and 13% O_2), no fertilized eggs reached the TS during the study. This resulted in non-convergence in generalized linear mixed models. In these cases, a dummy variable was substituted into the data set, consisting of one additional observation for each treatment that indicated that the embryo reached the TS on day 28. Data including a dummy variable were used with the generalized linear mixed model procedure and with the Cox Proportional Hazards procedure, but not to construct KM survival curves.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.C.V., H.J.B. and D.J. designed the experiment and analysis. The study was conducted by M.C.V. and A.M. M.C.V. analyzed the data and M.C.V. and D.J. wrote the manuscript. All authors approved the final manuscript.

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Movie 1. Hutchinsonian niche-space model depicting the conditions under which developing eggs reached threshold size. For details, see Fig. 5.

SUPPLEMENTARY TABLE

Table S1

Click here to Download Table S1

Table S2

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Table S3. Life tables for each of the 27 treatment conditions. Number failed is the number of fertilized eggs in each treatment that reached the threshold size of 5 mm^2 (TS). Number censored is the number of fertilized eggs that did not reach TS by the end of the study. Number at risk is the number of fertilized eggs remaining in each treatment with the possibility of reaching TS. These data were used to create all Kaplan-Meier survival curves.

Treatment	Day	Survival Function	Failure	SEM	Number Failed	Number Censored	At Risk
35 °C, 34 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	48
	14	0.81	0.19	0.06	9	1	48
	16	0.68	0.32	0.07	6	1	38
	20	0.64	0.36	0.07	2	0	31
	24	0.62	0.38	0.07	1	0	29
	28	0.57	0.43	0.07	2	26	28
30 °C, 34 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	48
	12	0.73	0.27	0.06	13	0	48
	14	0.25	0.75	0.06	23	0	35
	16	0.19	0.81	0.06	3	0	12
	28	0.19	0.81	0.06	0	9	9
25 °C, 34 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	48
	16	0.73	0.27	0.06	13	0	48
	18	0.27	0.73	0.06	22	0	35
	20	0.25	0.75	0.06	1	0	13
	22	0.23	0.77	0.06	1	0	12
	28	0.23	0.77	0.06	0	11	11
35 °C, 34 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	28	1.00	0.00	0.00	0	48	48
30 °C, 34 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	14	0.98	0.02	0.02	1	0	48
	16	0.54	0.46	0.07	21	0	47
	18	0.31	0.69	0.07	11	0	26
	20	0.29	0.71	0.07	1	0	15
	28	0.29	0.71	0.07	0	14	14
25 °C, 34 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	18	0.33	0.67	0.07	32	0	48
	20	0.17	0.83	0.05	8	0	16
	28	0.17	0.83	0.05	0	8	8
35 °C, 34 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	28	1.00	0.00	0.00	0	48	48

Table S3 continued

Treatment	Day	Survival Function	Failure	SEM	Number Failed	Number Censored	At Risk
30 °C, 34 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	22	0.96	0.04	0.03	2	0	48
	24	0.63	0.38	0.07	16	0	46
	26	0.25	0.75	0.06	18	0	30
	28	0.25	0.75	0.06	0	12	12
25 °C, 34 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	45
	22	0.71	0.29	0.07	13	0	45
	24	0.31	0.69	0.07	18	0	32
	26	0.22	0.78	0.06	4	0	14
	28	0.20	0.80	0.06	1	9	10
35 °C, 15 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	46
	10	0.91	0.09	0.04	4	0	46
	12	0.67	0.33	0.07	11	0	42
	14	0.57	0.43	0.07	5	0	31
	16	0.54	0.46	0.07	1	0	26
	18	0.50	0.50	0.07	2	0	25
	20	0.48	0.52	0.07	1	0	23
	28	0.48	0.52	0.07	0	22	22
30 °C, 15 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	47
	12	0.36	0.64	0.07	30	0	47
	14	0.23	0.77	0.06	6	0	17
	16	0.21	0.79	0.06	1	0	11
	28	0.21	0.79	0.06	0	10	10
25 °C, 15 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	48
	14	0.96	0.04	0.03	2	0	48
	16	0.48	0.52	0.07	23	0	46
	18	0.40	0.60	0.07	4	0	23
	20	0.38	0.63	0.07	1	0	19
	28	0.38	0.63	0.07	0	18	18
35 °C, 15 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	18	0.90	0.10	0.04	5	0	48
	20	0.88	0.13	0.05	1	0	43
	22	0.81	0.19	0.06	3	0	42
	24	0.79	0.21	0.06	1	0	39
	28	0.71	0.29	0.07	4	34	38
30 °C, 15 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	14	0.29	0.71	0.07	34	0	48
	16	0.21	0.79	0.06	4	0	14
	28	0.19	0.81	0.06	1	9	10

Table S3 continued

Treatment	Day	Survival Function	Failure	SEM	Number Failed	Number Censored	At Risk
25 °C, 15 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	16	0.81	0.19	0.06	9	0	48
	18	0.21	0.79	0.06	29	0	39
	24	0.19	0.81	0.06	1	0	10
	28	0.19	0.81	0.06	0	9	9
35 °C, 15 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	28	1.00	0.00	0.00	0	48	48
30 °C, 15 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	18	0.98	0.02	0.02	1	0	48
	20	0.75	0.25	0.06	11	0	47
	22	0.65	0.35	0.07	5	0	36
	24	0.54	0.46	0.07	5	0	31
	28	0.54	0.46	0.07	0	26	26
25 °C, 15 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	20	0.60	0.40	0.07	19	0	48
	22	0.21	0.79	0.06	19	0	29
	28	0.21	0.79	0.06	0	10	10
35 °C, 5 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	48
	28	1.00	0.00	0.00	0	48	48
30 °C, 5 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	46
	12	0.98	0.02	0.02	1	0	46
	14	0.78	0.22	0.06	9	0	45
	16	0.76	0.24	0.06	1	0	36
	18	0.74	0.26	0.06	1	0	35
	22	0.72	0.28	0.07	1	0	34
	28	0.72	0.28	0.07	0	33	33
25 °C, 5 ppt salinity, 21% O_2	0	1.00	0.00	0.00	0	0	48
	18	0.92	0.08	0.04	4	0	48
	20	0.77	0.23	0.06	7	0	44
	22	0.73	0.27	0.06	2	0	37
	28	0.73	0.27	0.06	0	35	35
35 °C, 5 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	28	1.00	0.00	0.00	0	48	48
30 °C, 5 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	18	0.77	0.23	0.06	11	0	48
	20	0.73	0.27	0.06	2	0	37
	24	0.71	0.29	0.07	1	0	35
	28	0.71	0.29	0.07	0	34	34

Table S3 continued

Treatment	Day	Survival Function	Failure	SEM	Number Failed	Number Censored	At Risk
25 °C, 5 ppt salinity, 13% O_2	0	1.00	0.00	0.00	0	0	48
	18	0.81	0.19	0.06	9	0	48
	20	0.67	0.33	0.07	7	0	39
	22	0.65	0.35	0.07	1	0	32
	24	0.60	0.40	0.07	2	0	31
	26	0.58	0.42	0.07	1	0	29
	28	0.58	0.42	0.07	0	28	28
35 °C, 5 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	28	1.00	0.00	0.00	0	48	48
30 °C, 5 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	22	0.98	0.02	0.02	1	0	48
	28	0.92	0.08	0.04	3	44	47
25 °C, 5 ppt salinity, 5% O_2	0	1.00	0.00	0.00	0	0	48
	28	0.71	0.29	0.07	14	34	48