



# Brief Episodes of Nocturnal Hypoxia and Acidification Reduce Survival of Economically Important Blue Crab (*Callinectes sapidus*) Larvae

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Many shallow coastal systems experience diel fluctuations in dissolved oxygen (DO) and pH that can intensify throughout the summer season and expose estuarine organisms to repeated episodes of coastal hypoxia and acidification. In temperate regions, larval release of the economically important blue crab *Callinectes sapidus* occurs in the summer, and while the earliest stage (zoea I) larvae are susceptible to persistent low DO and low pH conditions, their sensitivity to diel fluctuations is unknown. Here, a series of short-term ( $\leq 96$  h) experiments were conducted to investigate the survival of *C. sapidus* zoea I larvae exposed to a range of diel cycling hypoxic and acidified conditions and durations. Two experiments comparing a diel cycling DO/pH treatment (fluctuating from  $\sim 30\%$  air saturation to  $\sim 103\%$  averaging  $\sim 66\%$  and from pH  $\sim 7.26$  to  $\sim 7.80$  averaging  $\sim 7.53$ ) to a static low DO/pH treatment ( $\sim 43\%$ / $\sim 7.35$ ), a static moderate DO/pH treatment ( $\sim 68\%$ / $\sim 7.59$ ), and a static control treatment ( $\sim 106\%$ / $\sim 7.94$ ) indicated that survival in the diel cycling treatment was significantly lower than the moderate treatment ( $p < 0.05$ ) by 75 and 48% over 96 and 48 h, respectively, despite comparable mean experimental DO/pH values. Three other experiments aimed at identifying the effective minimum duration of low DO/low pH to significantly depress larval survival under diel cycling conditions revealed that 8 h of low DO/low pH ( $\sim 28\%$ / $\sim 7.43$ ) over a 24-h diel cycle consistently decreased survival ( $p < 0.05$ ) relative to control conditions by at least 55% regardless of experimental duration (72-, 48-, and 24-h experiments). An increase in DO beyond saturation to supersaturation (160%) and pH beyond normocapnic to highly basified (8.34) conditions during the day phase of the diel cycle did not improve survival of larvae exposed to nocturnal hypoxia and acidification. Collectively, these experiments demonstrate that diel cycling does not provide newly hatched *C. sapidus* larvae a temporal refuge capable of ameliorating low DO/pH stress, but rather is more lethal than chronic exposure to comparable average DO/pH conditions. Given that larvae exposed to a single nocturnal episode of moderate hypoxia and acidification experience significantly reduced survival, such occurrences may depress larval recruitment.

**Keywords:** hypoxia, coastal acidification, diel cycling, coastal change, larval release, blue crab, *Callinectes sapidus*

## INTRODUCTION

Covarying, diel fluctuations of dissolved oxygen (DO) and pH commonly occur across shallow estuaries worldwide. Driven by the oscillating dominance of photosynthesis and respiration over a diel cycle, shallow productive coastal systems experience naturally dynamic DO, pH, and CO<sub>2</sub> concentrations (Yates et al., 2007; Baumann et al., 2015; Baumann and Smith, 2017). Yet, many of these systems are additionally influenced by land-based practices that amplify the flux of nutrients and organic matter to coastal waters and accelerate metabolic processes (Nixon, 1995; Caffrey, 2004). Cultural eutrophication, or the increasing supply of organic matter to an ecosystem that is caused by anthropogenic influences such as nutrient enrichment (Malone and Newton, 2020), can intensify the amplitude of diel cycling and elicit repeated, severe episodes of nocturnal hypoxia as well as coastal acidification, particularly during warmer months when metabolic rates are maximal (Breitburg, 1990; Beck and Bruland, 2000; Baumann and Smith, 2017). Owing to the shallow nature of these systems, resident biota can be exposed to contemporaneous low DO and low pH conditions regardless of their position in the water column (Burnett, 1997; Tyler et al., 2009).

Biological responses to fluctuating hypoxia and/or acidification vary across estuarine taxa and life stages. Some early life stage fish species that utilize coastal habitat suffer reduced hatching success, growth, and/or survival, or engage in riskier behaviors that leave them more vulnerable to predation under diel cycling conditions relative to static ambient conditions (Davidson et al., 2016; Dixon et al., 2017; Morrell and Gobler, 2020), while others are quite tolerant (Lifavi et al., 2017; Targett et al., 2019) or only experience harmful effects under elevated temperatures (Targett et al., 2019). In some cases, diel cycling provides fish species a physiological refuge from, or reduction in negative behavioral effects compared to, chronically low DO and/or low pH conditions (Jarrold et al., 2017; Jarrold and Munday, 2018a; Cross et al., 2019), a benefit that can be temperature dependent (Laubenstein et al., 2020) or independent (Jarrold and Munday, 2018b). Field studies with oysters revealed significant negative differences in growth and immunocompromise controlled by diel cycling hypoxia/acidification frequency and magnitude, respectively (Breitburg et al., 2015), and *in situ* measures of heartbeat rates indicated that the metabolic activity of bay scallops increases and becomes more dynamic under diel cycling hypoxia (Gurr et al., 2018). Lab studies suggest additional, negative outcomes of diel cycling hypoxia and acidification among many mollusks across larval and juvenile life stages with increased infection prevalence by parasitic protozoan *Perkinsus* spp. (Keppel et al., 2015), and increased percent of time spent closed (Porter and Breitburg, 2016), as well as reductions in growth and survival (Clark and Gobler, 2016; Gobler et al., 2017) relative to ambient conditions. Studies of diel cycling impacts among crustaceans are limited, but some effects can be deduced from short term exposures (<24 h) to hypoxia. Sensitivities to short term exposures vary at the species level among decapod crustaceans, with juveniles of some species exhibiting significant lethargy and mortality over 24-h

(Eriksson and Baden, 1997) and 2-h exposures (Gravinese, 2020) to DO levels of ~25% air saturation.

The Atlantic blue crab, *Callinectes sapidus*, a species comprising one of the largest fisheries for the eastern United States (National Marine Fisheries Service (NMFS), 2019), is sometimes considered a climate change “winner” under future global change (Glandon et al., 2019). Rising coastal temperatures increase juvenile growth rates in higher latitude temperate systems (Brylawski and Miller, 2006; Glandon and Miller, 2017) and are projected to benefit Chesapeake Bay populations due to reductions in the duration of winter and associated juvenile mortality (Glandon et al., 2019). Juvenile and adult animals both exhibit steady or increased calcification and growth under acidified conditions (Ries et al., 2009; Whiteley, 2011; Glandon and Miller, 2017) and are also considered moderately tolerant of hypoxia (deFur et al., 1990; Burnett, 1997; Mangum, 1997). Recent experiments with larvae, however, reveal sensitivities of early life stages to ocean acidification (Giltz and Taylor, 2017) and coastal hypoxia and acidification both individually and in combination (Tomasetti et al., 2018), further evidencing that crustacean tolerance to low DO- and/or low pH-stress varies throughout ontogeny often in association with changes in respiratory physiology (Whiteley, 2011; Alter et al., 2015; Leiva et al., 2018; Gravinese, 2020).

In temperate systems, the *C. sapidus* larval release coincides with the time of year when coastal hypoxia/acidification is the most persistent and severe (Dittel R and Epifanio, 1982; McConaugha et al., 1983; Breitburg, 1990; Wallace et al., 2014). Among crustaceans, the pelagic larval life stage is considered the most sensitive to low DO (Miller et al., 2002), particularly for *C. sapidus*, for which the 50% lethal DO threshold (LC<sub>50</sub>) is higher than the majority of values published for other larval crustacean species (Miller et al., 2002; Tomasetti et al., 2018). Predictably, low DO effects on *C. sapidus* are most acute in the larval stage, as crustacean sensitivities to hypoxia depend on their respiratory anatomy and habitat characteristics (Alter et al., 2015). While zoeal development of *C. sapidus* occurs primarily in oxygenated surface shelf waters (Epifanio and Garvine, 2001), newly hatched first stage (zoea I) larvae can spend their first hours to days in estuarine or nearshore coastal habitat (Tagatz, 1968; Sandifer, 1973; Provenzano et al., 1983; Roman and Boicourt, 1999), where they may be exposed to harmful conditions. Survival of zoea I larvae is reduced significantly under low DO and combined low DO/low pH conditions over *static* 4-day exposures, with nearly all larvae perishing at DO concentrations  $\leq 2.5$  mg L<sup>-1</sup> or 35% (Tomasetti et al., 2018). However, many shallow eutrophic estuaries across the species' geographic range commonly experience large fluctuations in DO and pH during summer months (Tyler et al., 2009; Baumann et al., 2015; Baumann and Smith, 2017), and the larval sensitivities to diel cycling low DO and low pH exposures are unknown.

Therefore, this study presents a series of short-term experiments to investigate the survival of *C. sapidus* zoea I larvae to diel cycling hypoxia and acidification over a range of magnitudes and durations. Specifically, varied quantities of air, CO<sub>2</sub> and N<sub>2</sub> were delivered either continuously to create

distinct static DO and pH conditions or intermittently to create diel cycling DO and pH conditions and test our hypothesis that exposure to diel cycling hypoxia and acidification would negatively impact survival. Additional experiments altering the durations of hypoxia and acidification as well as the amplitudes of the changes in DO and pH under diel cycling were conducted to determine the relative importance of low DO/pH exposure durations and DO/pH fluctuations in driving larval mortality.

## MATERIALS AND METHODS

### Animal Collection and Maintenance

The effects of diel cycling hypoxia and acidification were examined through laboratory experiments exposing zoea I larvae to a range of static or cycling DO and pH values. All experiments were completed during June–August of 2019 and 2020 at the Stony Brook Marine Science Center in Southampton, New York. Ovigerous female blue crabs were retrieved from Shinnecock Bay, NY, United States (40.869415, –72.489203) as granted by a Scientific Collectors Permit via the New York State Department of Environmental Conservation. Individual females were placed in separate 20-L polyethylene buckets located in an 1890-L tank with continuous flow of mechanically (foam fractionated) and biologically (via fixed bed aerated biotowers) filtered recirculating UV sterilized sea water. The 20-L buckets were outfitted with openings (~7 cm in diameter) covered by 70  $\mu\text{m}$  mesh to allow exchange with filtered sea water but retain larvae. Individual buckets were aerated with ambient air via plastic tubing (Tygon) and glass bonded silica air stones until larval hatching. Newly hatched larvae were concentrated in a 2-L polypropylene beaker by siphoning the filtered sea water and larvae out from the lower half of the 20-L polyethylene buckets with plastic tubing through 1,000  $\mu\text{m}$  mesh that trapped egg mass strands and other particulates. Larvae were then distributed among experimental 1-L polypropylene vessels at densities that varied slightly between experiments but never exceeded 115 ind.  $\text{L}^{-1}$ , as greater densities (> 140 ind.  $\text{L}^{-1}$ ) can impair survival (Zmora et al., 2005) and are environmentally unrealistic (McConaughy et al., 1983). Larvae were fed live rotifers (50 ind.  $\text{ml}^{-1}$   $\text{day}^{-1}$ ) enriched with the algae *Nannochloropsis* originally grown in f/2 media (Sulkin and Epifanio, 1975; Zmora et al., 2005).

### Experimental Design

Five separate larval survival experiments were conducted, two comparing static DO/pH treatments to a diel cycling treatment, one comparing different durations of low DO/pH over diel cycles, and two comparing different amplitudes of diel cycling as well as different durations of low DO/pH over diel cycles (Table 1). Experiments varied in length (experimental duration), experiment type, starting larval density, number of females providing larvae, and treatments as described in Table 1. All DO and pH values and diel cycling durations and amplitudes were based on conditions found in US east coast estuaries and for most cases directly measured in two shallow lagoonal estuaries along the coast of NY hosting ovigerous female blue crabs during the summer larval hatching season (Figure 1; Tyler et al.,

2009; Baumann et al., 2015; Baumann and Smith, 2017). Briefly, multiparametric EXO2 sondes (Yellow Springs Instruments) were deployed during the summer of 2020 at two well-mixed estuaries with robust blue crab fisheries: Georgica Pond and Shinnecock Bay, to measure temperature, salinity, DO and  $\text{pH}_{\text{NBS}}$  (NBS scale) at a frequency of 10 min, in addition to an Ion Sensitive Field Effect Transistor (ISFET)-based solid state  $\text{pH}_{\text{T}}$  (total scale) SeaFet sensor (Satlantic) that was deployed alongside the multiparametric sonde in Shinnecock Bay to provide  $\text{pH}_{\text{T}}$  measurements (Figure 1). In Georgica Pond the sensor array was deployed on a surface buoy platform and all observations were made at a depth of 0.7 m regardless of sea surface height. In Shinnecock Bay the multiparametric sonde and ISFET pH sensor were affixed atop a large cinder block and placed on the sandy sediment (~0.3 m height above the bottom), but because Shinnecock Bay is a shallow, well-mixed tidal lagoon with an average depth of <2 m, observations were made at a depth of  $1.0 \pm 0.3$  m (mean  $\pm$  SD) and were representative of the pelagic environment. For all experiments, salinities were 27.6–29.9, and circulating water bath temperatures were maintained between 24 and 26°C (within the range of temperatures observed at Shinnecock Bay) by multiple heating wands (Finnex Aquarium Heaters) and an external water pump (Iwaki MD55RLT), conditions ideal for larval development (Costlow and Bookhout, 1959). When experiments exceeded 48 h, water changes occurred after 2 days, in which larvae were poured carefully onto a 250  $\mu\text{m}$  sieve and transferred into a glass dish of filtered seawater, before being returned to clean experimental vessels with new filtered seawater. Survival was quantified at the end of each experiment and recovered individuals were considered dead if they had undergone decay and/or did not respond with any movement to light from a flashlight (larvae move toward the region of greatest light intensity) after periods of > 30 s (Sandoz and Rogers, 1944).

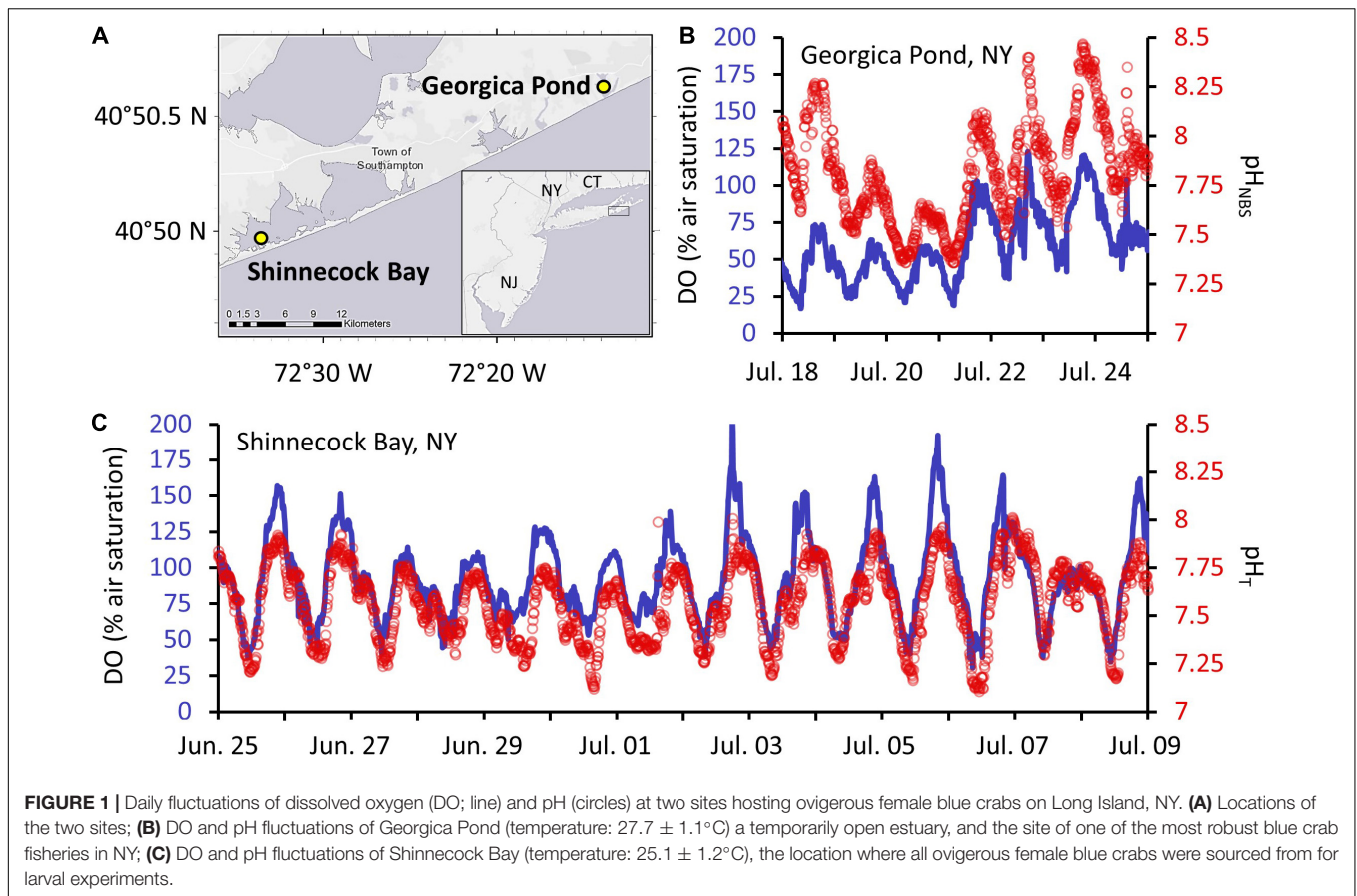
### Static vs Diel Cycling DO/pH Experiments

Two separate experiments comparing static DO/pH levels to diel cycling hypoxia/acidification were conducted that were similar in all aspects of experimental design but differed in experimental duration (experiment 1: 96-h total duration, experiment 2: 48-h total duration; Table 1). For both experiments, sixteen 1-L polyethylene vessels containing 0.2  $\mu\text{m}$  filtered seawater from Shinnecock Bay were placed in a sea table temperature-controlled water bath. Replicate ( $n = 4$ ) vessels received gas mixtures of ambient air,  $\text{N}_2$ , and  $\text{CO}_2$  at different rates to create and maintain either normoxic and normocapnic conditions [DO: ~106% air saturation (~7.4  $\text{mg O}_2 \text{ L}^{-1}$ )/pH: ~7.94; control treatment], moderately hypoxic and acidified conditions [~68% (~4.7  $\text{mg O}_2 \text{ L}^{-1}$ )/~7.59; moderate treatment], hypoxic and acidified conditions [~43% (~3.0  $\text{mg O}_2 \text{ L}^{-1}$ )/~7.35; low treatment] and diel cycling conditions (diel cycling treatment) that fluctuated between 12 h of hypoxic/acidified conditions [~30% (~2.0  $\text{mg O}_2 \text{ L}^{-1}$ )/~7.26] at night and 12 h of normoxic/normocapnic conditions [~103% (~7.2  $\text{mg O}_2 \text{ L}^{-1}$ )/~7.80] during the day, creating average DO and pH values [~66% (~4.6  $\text{mg O}_2 \text{ L}^{-1}$ )/~7.53] comparable to the moderate treatment. The target

**TABLE 1** | Overview of experimental conditions for five experiments investigating the survival of *Callinectes sapidus* zoea I larvae to diel cycling hypoxia and acidification.

Experiment number	Experiment type	Experiment duration (h)	Initial density (ind. L <sup>-1</sup> )	Larvae source	Treatments static (s) fluctuating (f)	Diel cycling duration (h of low DO and low pH 24 h <sup>-1</sup> )	Diel cycling amplitude: [range of DO (% air sat) and pH values 24 h <sup>-1</sup> ]
1	Static vs diel cycling	96	104	1 mother	Control (s)	–	–
					Moderate (s)	–	–
					Low (s)	–	–
					Diel cycling (12-h) (f)	12	Moderate DO: 29–104% pH: 7.21–7.78
2	Static vs diel cycling	48	112	3 mothers	Control (s)	–	–
					Moderate (s)	–	–
					Low (s)	–	–
					Diel cycling (12-h) (f)	12	Moderate DO: 30–101% pH: 7.31–7.82
3	Diel cycling duration	72	114	3 mothers	Control (s)	–	–
					4-h (f)	4	Moderate DO: 34–99% pH: 7.34–7.90
					8-h (f)	8	Moderate DO: 32–100% pH: 7.36–7.92
					12-h (f)	12	Moderate DO: 31–97% pH: 7.33–7.88
4	Diel cycling duration and amplitude	48	92	1 mother	Control (s)	–	–
					4-h (f)	4	Moderate DO: 27–99% pH: 7.45–8.00
					8-h (f)	8	Moderate DO: 30–99% pH: 7.41–7.99
					12-h (f)	12	Moderate DO: 29–97% pH: 7.39–7.98
					12-h SC (f)	12	Strong DO: 30–160% pH: 7.44–8.34
5	Diel cycling duration and amplitude	24	95	1 mother	Control (s)	–	–
					4-h (f)	4	Moderate DO: 25–97% pH: 7.54–8.04
					8-h (f)	8	Moderate DO: 27–99% pH: 7.54–8.06
					12-h (f)	12	Moderate DO: 25–99% pH: 7.47–8.07
					12-h WC (f)	12	Weak DO: 25–66% pH: 7.48–7.63





levels in the static low treatment differed from the target low values in the diel cycling treatment because previous experiments established that 100% larval mortality was common at  $\sim 30\%$  ( $\sim 2.0 \text{ mg O}_2 \text{ L}^{-1}$ ) over static 96-h exposures (Tomasetti et al., 2018) an outcome we tried to avoid. To achieve the diel cycling, solenoid valves were attached to a timer (Hunter) programmed to regulate the flow of tanked gas mixtures bubbled into the seawater for 12 h at night followed by air for 12 h during the day (Clark and Gobler, 2016; Morrell and Gobler, 2020).

## Diel Cycling Duration and Amplitude Experiments

Three additional larval survival experiments compared various durations of low DO/pH within diel cycling conditions (experiment 3: 72-h experiment) or compared both durations of low DO/pH and amplitudes of diel cycling (experiments 4 and 5: 48- and 24-h experiments, respectively; **Table 1**). For all experiments, replicate ( $n = 4$ ) vessels were exposed to static normoxic and normocapnic conditions [DO:  $\sim 99\%$  ( $\sim 6.9 \text{ mg O}_2 \text{ L}^{-1}$ )/pH:  $\sim 7.97$ ; control treatment], or fluctuating low DO/pH exposures [ $\sim 28\%$  ( $\sim 2.0 \text{ mg O}_2 \text{ L}^{-1}$ )/ $\sim 7.43$ ] that had durations of low DO/pH lasting for 4 h (4-h treatment), 8 h (8-h treatment), or 12 h (12-h treatment) during the night, before cycling back to the day phase [ $\sim 99\%$  ( $\sim 6.9 \text{ mg O}_2 \text{ L}^{-1}$ )/ $\sim 7.97$ ] for the remainder of the daily 24-h cycle. All treatments with fluctuating DO/pH values were established through regulating gas mixtures

through solenoid valves controlled by timers (Hunter) that varied in duration (4 vs 8 vs 12 h) based on the treatment.

The final two experiments investigating low DO/pH durations (experiments 4 and 5) also compared differences in amplitudes of diel cycling (**Table 1**). When exploring different amplitudes of diel cycling, we considered that intense photosynthetic activity in shallow environments can supply additional DO (increasing DO beyond saturated to supersaturated levels) and consume substantial quantities of  $\text{CO}_2$  (increasing pH beyond normocapnic to basified levels) in the day phase of a diel cycle (Breitburg, 1990; Tyler et al., 2009; Baumann et al., 2015). Alternatively, the day phase of diel cycling may only partially alleviate hypoxic/acidified conditions, meaning DO and pH values do not reach 100% saturation and normocapnia, respectively (Breitburg, 1992). To compare the effects of different amplitudes of diel cycling on larval survival, another treatment ( $n = 4$  replicates) was added to simulate strong (12-h strong cycling amplitude treatment; experiment 4) or weak (12-h weak cycling amplitude treatment; experiment 5) diel cycling amplitudes (**Table 1**). To establish the 12-h strong cycling amplitude treatment in experiment 4, nitrox gas (65%  $\text{N}_2$ , 35%  $\text{O}_2$ ) combined with  $\text{CO}_2$ -stripped air (Keppel et al., 2016; Porter and Breitburg, 2016; Giomi et al., 2019) was introduced in place of ambient air for 12-h durations during the day phase of the 24-h diel cycle to create supersaturated and basified conditions [ $160\%$  ( $11.3 \text{ mg O}_2 \text{ L}^{-1}$ )/8.34]. The 12-h weak cycling amplitude treatment of experiment 5, rather than receive solely ambient

air or nitrox gas during the 12 h day phase, received a different combination of N<sub>2</sub>, CO<sub>2</sub>, and air for 12 h, to create DO/pH conditions of 66% (4.7 mg O<sub>2</sub> L<sup>-1</sup>)/7.63. For both experiments, the night phase low DO/pH values remained identical across all diel cycling treatments (Table 1).

## Chemistry

Temperature, DO, and pH<sub>T</sub> measurements were made twice daily for all static and fluctuating (once during the night phase and once during the day phase) treatments using a calibrated optical DO meter (Yellow Springs Instruments, ProODO) and a Honeywell Durafet ISFET-based pH<sub>T</sub> sensor calibrated with Dr. Andrew Dickson's (Scripps Institute of Oceanography) seawater pH standard, respectively. To calculate the entire suite of carbonate chemistry parameters for each experiment, seawater samples were collected from replicates across each treatment at the beginning and/or end of the experiment in borosilicate, biological oxygen demand (BOD) bottles, preserved with 1% mercuric chloride, and sealed with a glass stopper using Apiezon L ultra-high vacuum grease and rubber bands, for dissolved inorganic carbon (DIC) analyses (Riebesell et al., 2011). A VINDTA 3D (Versatile Instrument for the Determination of Total Carbon) delivery system extracted DIC from samples which was released as CO<sub>2</sub> to a UIC CM5017O coulometric titration unit that measured total DIC. Certified reference materials (CRM) provided by Dr. Andrew Dickson (Scripps Institute of Oceanography) were analyzed before each set of analyses, which only proceeded when recovery of CRM exceeded 99.6%. For each treatment, all carbonate chemistry parameters were calculated via the CO2SYS program<sup>1</sup> using measured values of pH, DIC, temperature, salinity, pressure, and H<sub>2</sub>CO<sub>3</sub> dissociation constants (Millero, 2010).

## Statistical Analyses

RStudio statistical software (Version 1.3.959) was used for all statistical analyses, and the map was created with ArcMap (10.7.1). Differences in percent survival data were assessed using Analysis of Variance (ANOVA) tests with treatment as the explanatory variable, followed by *post hoc* Tukey Honest Significant Difference tests. Shapiro-Wilk tests of normality and Bartlett's tests for homogeneity of variance were conducted to test ANOVA assumptions, and if an assumption was not met survival data was arc-sin square root transformed and retested. All survival values are reported as means ± standard error. Seawater chemistry values are reported as means ± standard deviations, with temperature, DO and pH values derived from twice daily measurements of these parameters in all replicates per treatment, and all other carbonate chemistry parameters derived from pH and DIC measurements from the replicates of each treatment at the start and/or end of experiments. Differences between average DO and pH levels in static and diel cycling treatments were assessed via Kruskal-Wallis ANOVAs followed by *post hoc* Wilcoxon rank sum tests, correcting for multiple comparisons through the Benjamini-Hochberg procedure.

<sup>1</sup>[https://cdiac.ess-dive.lbl.gov/ftp/oceans//co2sys/CO2SYS\\_calc\\_XLS\\_v2.3/](https://cdiac.ess-dive.lbl.gov/ftp/oceans//co2sys/CO2SYS_calc_XLS_v2.3/)

## RESULTS

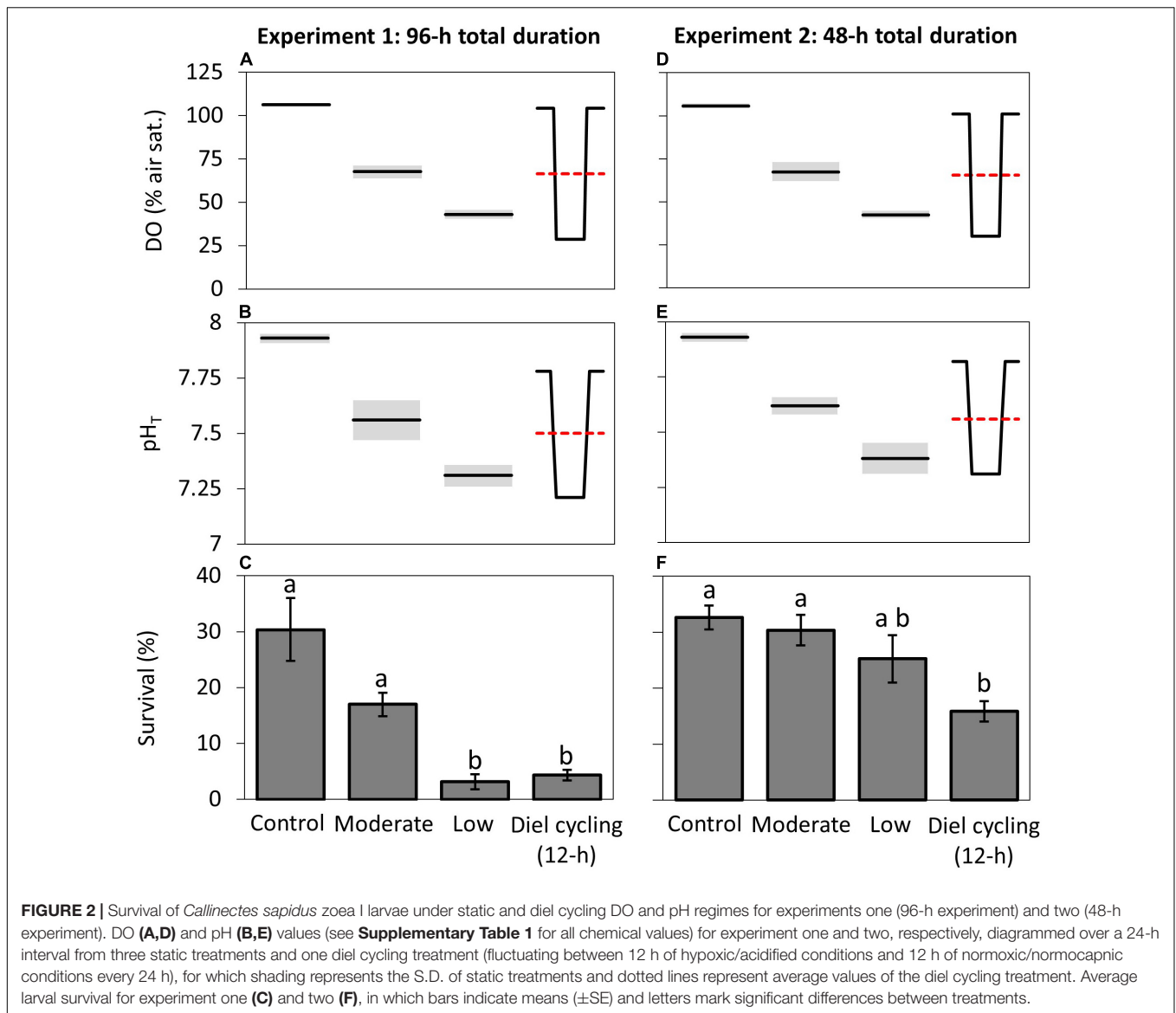
### Static vs Diel Cycling DO/pH Experiments

In the first and second experiments, *C. sapidus* zoea I larval survival was compared across static control, static moderate, static low, and diel cycling treatments (Figure 2, Table 1, and see Supplementary Table 1 for detailed chemistry). For both experiments average DO levels were significantly different across all static and diel cycling treatments except for between the moderate and diel cycling treatments, as intended ( $p < 0.05$ , Wilcoxon test; Figure 2). The average pH in the diel cycling treatment of experiment 1 had a slightly lower average pH value ( $7.50 \pm 0.05$ ) than the moderate treatment ( $7.56 \pm 0.02$ ) but were still comparable given the range of average pH conditions among the other static treatments (7.93 and 7.31), and the average pH in experiment 2 was statistically indistinguishable from the moderate treatment, as intended.

For experiment 1 (96 h), among the static treatments, survival in the low treatment ( $3 \pm 1\%$ ) was significantly lower ( $p < 0.005$ , Tukey HSD) than both the moderate ( $17 \pm 2\%$ ) and control ( $30 \pm 6\%$ ) treatments for which there were no significant differences in survival (Figure 2 and Supplementary Table 2). Exposure to diel cycling did not provide refuge from low DO/pH effects, as larval survival in the diel cycling treatment ( $4 \pm 2\%$ ) was significantly lower than the moderate and control treatments ( $p < 0.05$ , Tukey HSD), and statistically indistinguishable from the low treatment (Figure 2 and Supplementary Table 2). Given the differences observed in the first experiment and the low survival in the low and diel cycling treatments, the second experiment sought to observe responses to similar chemistry over a shorter time frame, 48 h. Unlike the first experiment, there were no significant differences in larval survival among the static control ( $33 \pm 2\%$ ), moderate ( $30 \pm 3\%$ ), and low ( $25 \pm 4\%$ ) treatments (Figure 2). The diel cycling treatment, however, exhibited significantly reduced survival ( $16 \pm 2\%$ ) relative to the control and moderate treatments ( $p < 0.05$ , Tukey HSD; Supplementary Table 2), while remaining statistically indistinguishable from the low treatment (Figure 2). This further emphasizes the failure of diel cycling to provide refuge from hypoxic- and acidification-stress to blue crab larvae.

### Diel Cycling Duration and Amplitude Experiments

Following the static vs diel cycling experiments presented above, three additional experiments of decreasing experimental duration (experiment 3: 72 h, experiment 4: 48 h, experiment 5: 24 h) were performed to identify the effective minimum duration of low DO/low pH needed to significantly depress larval survival (Table 1). These experiments compared the survival of *C. sapidus* zoea I larvae in static control conditions to that of diel cycling conditions that varied in the (i) duration of the low DO/pH conditions during the night phase (4 vs 8 vs 12 h), and for experiments four and five, (ii) the amplitude (moderate vs strong vs weak) of the diel cycling (Table 1 and Figure 3). For each experiment, the magnitude of the low DO/pH values were



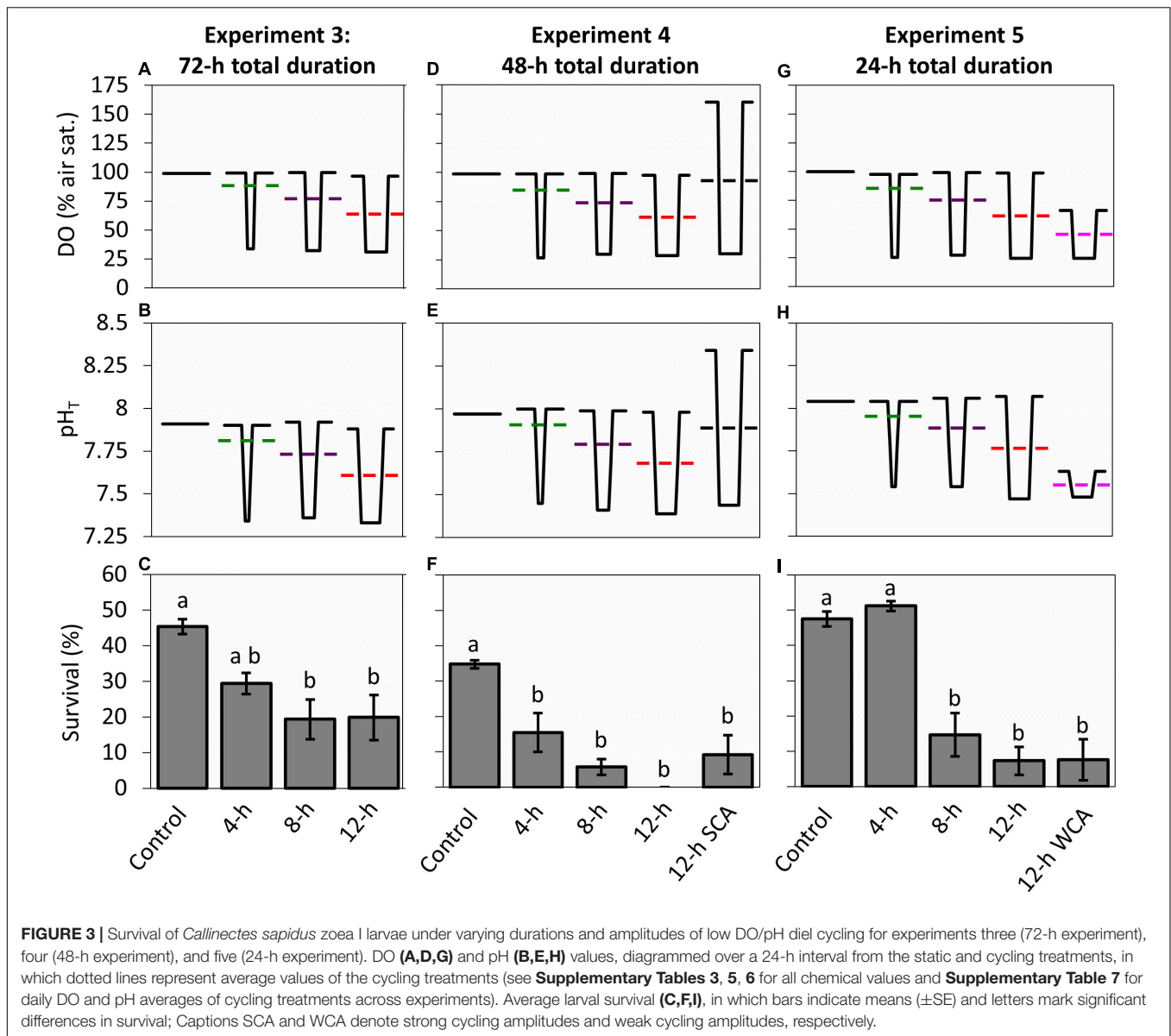
statistically indistinguishable across all diel cycling treatments, as intended ( $p > 0.05$ ; Kruskal-Wallis ANOVAs).

In the third experiment (72 h), DO/pH conditions were static for the control treatment and fluctuating between a low DO/pH phase in the night that lasted for 4, 8, or 12 h, and a high DO/pH phase that lasted the remainder of the 24-h cycle (Figure 3 and see **Supplementary Table 3** for detailed chemistry). Larval survivorship in the 8-h ( $19 \pm 6\%$ ) and 12-h treatments ( $20 \pm 6\%$ ), were significantly lower ( $p < 0.05$ , Tukey HSD) than the control treatment ( $45 \pm 2\%$ ), while the 4-h treatment ( $29 \pm 3\%$ ) was not (Figure 3 and **Supplementary Table 4**). There were no significant differences in larval survival among the cycling treatments.

During the fourth experiment (48 h), a 12-h strong cycling amplitude treatment was added, and the fourth experiment duration was also 24 h shorter (Figure 3, Table 1, and see **Supplementary Table 5** for detailed chemistry). Fluctuating DO/pH significantly reduced larval survival across all treatments

relative to the control ( $p < 0.05$ , Tukey HSD; **Supplementary Table 4**) with the survival of 4-h ( $15 \pm 6\%$ ), 8-h ( $6 \pm 2\%$ ), 12-h ( $0\%$ ), and 12-h strong cycling amplitude ( $9 \pm 5\%$ ) treatments all  $< 50\%$  of the control ( $35 \pm 1\%$ ) treatment (Figure 3). Like the prior experiment, there were no significant differences across cycling treatments (Figure 3). Additionally, the amplitude of the diel cycle did not significantly impact larval survival (Figure 3). Due to the complete mortality across all replicates of the 12-h treatment, the homogeneity of variance ANOVA assumption was not met, however, ANOVA is robust to violations of the equal variance assumption if the sample sizes are equal (Boneau, 1960).

In the final experiment which persisted for only 24 h (experiment 5), a new treatment—the 12-h weak cycling amplitude treatment—replaced the 12-h strong cycling amplitude treatment of the prior experiment (Figure 3, Table 1, and see **Supplementary Table 6** for detailed chemistry). Larval survival in the 8-h ( $15 \pm 6\%$ ), 12-h ( $7 \pm 4\%$ ), and 12-h weak cycling



amplitude ( $8 \pm 6\%$ ) treatments were significantly lower than the control ( $48 \pm 2\%$ ) treatments ( $p < 0.005$ , Tukey HSD), but contrary to the previous two experiments, were also significantly lower than the 4-h ( $51 \pm 1\%$ ) treatment ( $p < 0.005$ , Tukey HSD; **Figure 3** and **Supplementary Table 4**). There were no significant differences between the control and 4-h treatments, and again the amplitude of diel cycling did not impact larval survival (**Figure 3**).

## DISCUSSION

Complex interactions between global and smaller scale biogeochemical processes can create highly dynamic coastal environmental conditions (Tyler et al., 2009; Rabalais et al., 2010; Carstensen and Duarte, 2019; Rheuban et al., 2019). The dynamic

range of DO, CO<sub>2</sub>, and pH in shallow eutrophic estuaries over diel cycles can force organisms to endure environmental extremes for multiple hours each day. This study revealed that brief exposures to coastal hypoxia and acidification under fluctuating DO/pH regimes consistently elicited significant mortality of *C. sapidus* zoea I larvae. Daily DO and pH values of  $\sim 66\%$  ( $\sim 4.6$  mg O<sub>2</sub> L<sup>-1</sup>) and  $\sim 7.59$ , conditions that are commonly observed across eastern US estuaries (Baumann and Smith, 2017), while benign under *static* exposures, induced precipitous declines in survival under *diel cycling* exposures, decreasing survival by as much as 75% in the diel cycling treatment compared to the static moderate treatment over a 4-day period (experiment 1). Indeed, significant mortality ( $>90\%$ ) occurred in fluctuating treatments with daily DO and pH averages as high as 95% ( $6.7$  mg O<sub>2</sub> L<sup>-1</sup>) and 7.89, conditions common in most temperate and tropical estuaries. Despite the



daily restoration of favorable environmental conditions, diel cycling did not provide larvae a refuge from the physiological stress associated with chronically low DO/pH conditions, even when daytime DO and pH levels were supersaturated and basified, respectively, and even when exposures were as short as one 8-h period.

The physiological capacity to maintain homeostasis in dynamic DO/CO<sub>2</sub> environments likely depends on both the adaptive history and life stage of the animal (Waldbusser and Salisbury, 2014; Alter et al., 2015; Leiva et al., 2018). Many early-stage decapod planktonic larvae are unable to regulate their metabolism under declining DO (Belman and Childress, 1973; Spicer, 1995). Their rudimentary scaphognathites (appendages used to draw water toward the gills) and absence of functional gills (Costlow and Bookhout, 1959) suggests that gas exchange occurs across the body's surface, severely restricting decapod larvae from altering the rate of O<sub>2</sub> uptake and CO<sub>2</sub> expulsion (Anger, 2001). Eight or twelve consecutive hours of hypoxia [ $\sim 28\%$  ( $\sim 2.0$  mg O<sub>2</sub> L<sup>-1</sup>)] under diel cycling regimes induced significant mortality (>80%) across all experiments, regardless of the experimental duration, including over only 1 day. In another study, megalopae, which exhibit more developed respiratory morphology and a greater ability to oxyregulate, experienced >80% mortality when exposed to eighteen sustained hours of 20% air saturation ( $\sim 1.4$  mg O<sub>2</sub> L<sup>-1</sup> at the temperature/salinity of our experiments), suggesting slightly improved tolerances compared to zoea I larvae (Tankersley and Wieber, 2000). Adults, by contrast, can endure over 350 h of hypoxia [34% (2.46 mg O<sub>2</sub> L<sup>-1</sup>)] and experience no significant mortality relative to crabs in normoxic conditions (Brouwer et al., 2005; Brown-Peterson et al., 2005). Curiously, first-instar juveniles (J1s) were shown to deviate from the pattern of increased hypoxia tolerance with age, as J1 individuals were more vulnerable than megalopae (Tankersley and Wieber, 2000), perhaps as a result of the energetic costliness of the molting process associated with transition to the J1 stage.

Several behavioral, morphological, and physiological adaptations have been identified that support the general trend of increased hypoxia tolerance with age in the blue crab. Firstly, juvenile and adult blue crabs can simply relocate away from hypoxic habitat (Pihl et al., 1991). Additionally, the development of functional scaphognathites and structurally complex gills among benthic-stage blue crabs permits more efficient O<sub>2</sub> extraction under declining DO conditions through hyperventilation (Batterton and Cameron, 1978; McMahon and Wilkens, 1983). And physiologically, hypoxia can induce increases in O<sub>2</sub> affinity among adult blue crabs by increasing the amount of, and stimulating a structural change to, the respiratory protein hemocyanin (deFur et al., 1990; Mangum, 1997). Adaptations that benefit adult blue crabs under hypoxia can also increase tolerance to coastal acidification. Ion exchange across the gills, for example, allows for regulation of HCO<sub>3</sub><sup>-</sup> in the hemolymph (Cameron, 1985) that, in addition to hemocyanin (Whiteley, 2011), buffers hemolymph pH (Melzner et al., 2009). Moreover, for adult blue crabs, the high CO<sub>2</sub> concentrations commonly found in hypoxic environments can actually mitigate exercise fatigue (lengthening time until fatigue and reducing

the number of turns and stops while walking) under hypoxia (Stover et al., 2013) by increasing hemocyanin oxygen binding affinity (Mangum and Burnett, 1986; Lehtonen and Burnett, 2016), an adaptation that improves blue crab mobility and the ability to ultimately escape low DO/low pH conditions (Lehtonen and Burnett, 2016).

Lacking many of these adaptations at the zoea I stage, *C. sapidus* larvae exhibited rapid mortality over relatively short (24–96 h) timescales. Consistent across all experiments, significant negative effects were detected in the diel cycling treatments with 8 or 12 h of low DO/pH, whereas effects of 4-h treatments varied depending on the experiment. Eight hours of stress over 1 day induced significantly increased mortality (experiment 5), for instance, yet 4 h of stress each day for three consecutive days did not (experiment 3). Hence, the lethal threshold for daily exposure to comparable levels of low DO/pH is likely somewhere between 4 and 8 h, above which the metabolic strains of the high O<sub>2</sub> demand and limited supply overwhelm the larvae. Respiration rates of early stage decapod larvae often decrease substantially under low partial pressures of O<sub>2</sub> (Anger, 2001), indicating that the low availability of O<sub>2</sub> over 8-h or longer durations can limit energy metabolism and prevent larvae from maintaining energy balance. Yet 4-h exposures can significantly reduce larval survival at times, as demonstrated in experiment 4 (48-h total duration). Interestingly, the average larval survival of the ambient treatment in that experiment was the lowest of the three duration experiments, suggesting that larval health may have already been compromised or that maternal effects may contribute to short-term low DO/pH tolerance. Whenever possible we controlled for maternal effects by stocking single experiments with larvae from multiple mothers, however, when larval release was not coordinated or only one female was available the larvae were still used in experiments (Table 1).

While either nocturnal acidification, hypoxia, or a combination of the two could have contributed to decreased survival of crab larvae, we hypothesize that the low DO was the primary driver of mortality. Although the challenges of acid-base balance under low pH stress may act as a secondary stressor, Tomasetti et al. (2018) determined that significant acidification effects were not detected after 96 h of low pH similar to levels explored here but were detected after 14-day exposures. This was consistent with another study that found significant ocean acidification effects on *C. sapidus* larvae after periods exceeding 10 days and suggested that the energetic requirements of molting may have contributed to their sensitivity to low pH (Giltz and Taylor, 2017). Our short ( $\leq 96$  h) experiments ensured that all effects occurred days before the first molt (Costlow and Bookhout, 1959) and were not a result of increased vulnerability or energetic strain associated with the molting process. Nonetheless, our experiments intentionally did not separate the two stressors seeking instead to mimic their covariance, so it is possible that the reported negative effects on larval survival are the result of a combination of low DO and low pH stress, especially since fluctuating co-stressors had not previously been tested.

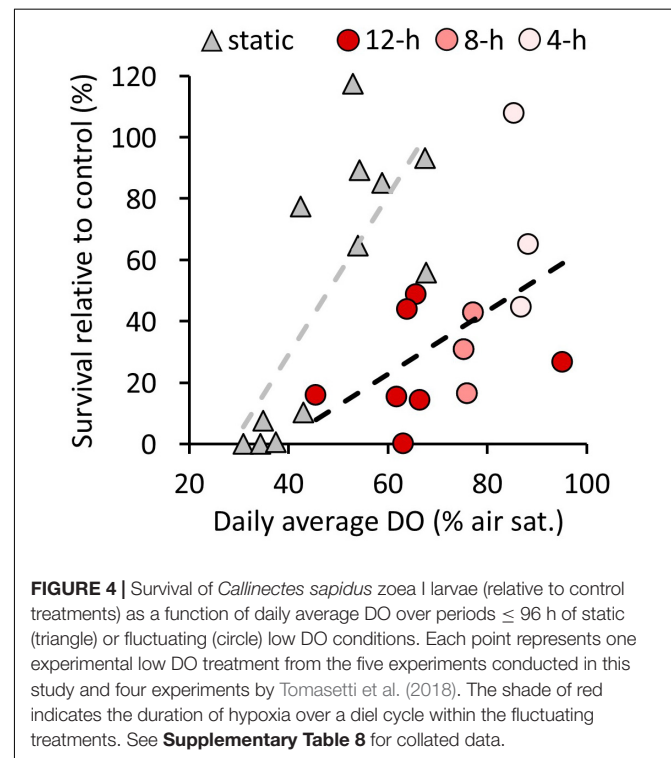
Although 12-h durations of diel cycling hypoxia/acidification were not directly measured during our environmental

monitoring, this severe scenario is not unrealistic for surface waters impacted by cultural eutrophication (Tyler et al., 2009; Wallace et al., 2014). This scenario may not apply to some systems such as some deep stratified systems or shallow oligotrophic systems, where shorter or less intense exposures may be more apt. Yet we note that for some systems, the true impacts of nocturnal hypoxia on *C. sapidus* larvae at times may be more severe than reported here as our experiments used low DO conditions  $\sim 28\%$  saturation or  $\sim 2.0 \text{ mg L}^{-1}$ ; shallow eutrophic estuaries can experience nocturnal DO concentrations near or at the surface that are much lower than this including anoxia (see Beck and Bruland, 2000, measured at a depth  $< 1.3 \text{ m}$ ; Tyler et al., 2009, measured throughout the entire water column; Wallace, 2020, measured at depths of 0.7–1.3 m at multiple locations). These findings have important implications for *C. sapidus* populations and fisheries/estuarine management, as even small reductions in larval mortality rates can markedly decrease post-dispersive recruitment (Underwood and Fairweather, 1989). Detection of zoea I larvae in waters with salinities as low as 16 (Sandifer, 1973) and more commonly in polyhaline salinity zones (Sandifer, 1975), as well as detection of zoea II larvae at the mouths of large embayments (Tagatz, 1968; Sandifer, 1973) suggests that larval release can sometimes occur upstream of estuary mouths, committing larvae to extended periods in the estuary. Weekly and/or monthly sampling in Delaware and Chesapeake Bays demonstrated that *C. sapidus* larval abundances peak in late summer (Sandifer, 1973; Dittel R and Epifanio, 1982; Epifanio et al., 1984) when coastal hypoxia/acidification is often most severe (Breitburg, 1990; Wallace et al., 2014). Moreover, larvae are released at night (Provenzano et al., 1983) or in the early morning (Tankersley et al., 2002), maximizing the likelihood of encountering the low DO/pH conditions associated with the most stressful phase of the diel cycle (Tyler et al., 2009; Baumann et al., 2015). Collectively, these observations suggest that estuarine DO and pH conditions could influence the success of larval recruitment to the juvenile stage.

Decades of larval transport research have established the general pattern of estuarine export and offshore development for *C. sapidus* larvae in the Mid-Atlantic (Epifanio, 1995, 2019; Epifanio and Garvine, 2001). Very little is known regarding patterns of larval release and transport north of Delaware Bay (e.g., for populations inhabiting NY and CT waters) where the coastal morphology, currents, and circulation patterns differ and where profits from blue crab landings have expanded in recent decades (National Marine Fisheries Service (NMFS), 2019). For example, NY and other northeast United States host a plethora of barrier island estuaries, some of which have ocean inlets that are entirely closed to the Atlantic Ocean during summer months (e.g., Mecox Bay, Sagaponack Pond, Georgica Pond, NY, United States; Gobler et al., 2005). In NY, these lagoonal estuaries host some of the most robust blue crab fisheries of the state. The life cycle of blue crabs in these systems is presently unknown as are the effects of the intense diel cycling hypoxia and acidification in these systems (Figure 1) on resident *C. sapidus* larvae. Additional environmental monitoring and larval transport research in these regions is needed to clarify the frequency and durations with which larvae of *C. sapidus* and other species encounter coastal hypoxia/acidification.

The acute sensitivities of *C. sapidus* larvae to coastal hypoxia revealed in our study are consistent with other meroplanktonic decapod species that inhabit well-oxygenated coastal surface waters for the majority of their larval development (Spicer, 1995; Alter et al., 2015). Specific adaptations such as catadromous migrations of adult females (Tankersley et al., 1998) and post-hatch upward swimming motion of larvae (Sulkin et al., 1980) likely make estuarine retention of *C. sapidus* over many diel cycles rare as larvae are eventually flushed out of estuaries to the shelf. Indeed, zoea I larvae outnumbered all other stages sampled in Chesapeake Bay, and are commonly found at more offshore stations in both Chesapeake and Delaware Bays (Sandifer, 1973, 1975; Epifanio et al., 1989). These patterns, however, may also reflect the challenges of surviving even short durations of estuarine transport, given the variable DO/pH regimes and high predation rates.

Despite their differences from *C. sapidus* larvae, estuarine species with fully estuarine larval life history strategies may not necessarily be adapted to low DO/low pH. Rather, they may merely be closer to specific tolerance-limits that will potentially be encountered more frequently or for longer durations under continued climate change (Waldbusser and Salisbury, 2014; Breitburg et al., 2018; Grear et al., 2020). Larval *Argopecten irradians*, *Mercenaria mercenaria*, and *Crassostrea virginica*, for instance, exhibited significant reductions in survival and growth under diel cycling hypoxia and acidification that were no less severe than under chronic stress (Clark and Gobler, 2016). Such larval sensitivities to diel cycling hypoxic/acidified conditions likely contribute to the challenges associated with shellfish restoration in eutrophic environments for which



shellfish populations often fail to recover to historical abundances (Jackson et al., 2001; Mann and Powell, 2007; Johnson et al., 2009).

## CONCLUSION

Natural and direct anthropogenic pressures influence the dynamic range of coastal DO and pH conditions and can result in short, repeated exposure to environmental extremes over diel and/or tidal cycles. In coastal environments subject to high dynamicity, the extreme conditions in some cases may be more relevant to the ecology of the system than average conditions. Pooling percent survival data from this study and experimental data from Tomasetti et al. (2018) where larvae were exposed to a range of static low DO levels for periods  $\leq 96$  h (by first time point) further evidenced that at comparable average DO values, diel cycling hypoxic conditions often elicited more severe reductions in larval survival than static treatments (**Figure 4** and **Supplementary Table 8**). For instance, static DO levels  $> 50\%$  air saturation never elicited declines in survival  $> 50\%$ , yet under diel cycling hypoxia  $> 50\%$  declines in survival were common at daily average DO levels ranging from 62 to 95%, occurring in 10 of 12 cases with the two exceptions involving 4-h exposures (**Figure 4**). Short ( $\leq 24$  h) exposures to low DO have been shown to negatively impact the survival of *C. sapidus* megalopae and J1 individuals that may experience these conditions when they return and settle in estuarine habitat (Tankersley and Wieber, 2000). Our novel findings suggest that reductions in recruitment may similarly be influenced by coastal hypoxia/acidification encountered at the zoea I stage via a significant increase in mortality rates among larvae exposed briefly (8 h) to low DO/pH conditions during estuarine export. Management plans for coastal fisheries that consider fluctuating environmental conditions may better support their long-term sustainability, particularly for estuarine species, as exposures to low DO/pH over timescales shorter than one tidal period were proven to be lethal here. Advancements in autonomous *in situ* monitoring technology can now provide more cost-effective long term, high frequency environmental data and can reveal episodes of hypoxia/acidification that manual measurements made during the hours of daylight would miss. Our results highlight the value of integrating coastal DO and carbonate system variability into experimental designs as static scenarios can conceal adverse outcomes. Average environmental conditions do not always provide the most relevant information to assess vulnerability to stressors in fluctuating environments, especially given the high sensitivity and short timescales of biological processes such as early shell formation or larval development. Consequently,

regulatory standards that simply rely on average DO and/or pH conditions rather than extremes or durations of extremes may leave aquatic life vulnerable to environmental harm, particularly under future coastal conditions (Tomasetti and Gobler, 2020). To address these uncertainties, additional studies examining the tolerances of coastal biota at early life stages to multiple stressors under various amplitudes and durations of fluctuating conditions are required.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

ST, JK, and CG conceived and designed the experiments, analyzed the data, and revised the manuscript. ST and JK performed the experiments. CG contributed the reagents, materials, and analysis tools. ST and CG wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.720175/full#supplementary-material>

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