

NOTE

Resilience of Black Sea Bass Embryos to Increased Levels of Carbon Dioxide

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Abstract

After a decade of research on how embryonic fish will respond to the increased dissolved carbon dioxide (ρCO_2) levels predicted for the next century, no uniform response to near future acidification has been observed among marine species. We exposed Black Sea Bass *Centropristis striata* (BSB) embryos to varied levels of ρCO_2 (microatmospheres [μatm]) for 48 h during seasonal experiments conducted in 2013–2015 to compare embryonic response among multiple broodstocks. The relationship between ρCO_2 concentration and hatching success was inconsistent among years, with a nonlinear, inverse relationship noted in 2014 only, explaining 13% of observed variance. Conversely, ρCO_2 was a good predictor of unhatched BSB embryos after 48 h for all years combined (39%) and for 2013 (38%). The ρCO_2 concentration was a good predictor of the frequency of vertebral column anomalies for individual years (2013: 40%; 2014: 12%; 2015: 38%) but not when data were pooled for all years. In 2013 and 2015, vertebral column anomalies were relatively consistent below 1,000 μatm and were elevated above that threshold. Preliminary results suggest that BSB embryos may demonstrate resilience to future ρCO_2 levels, but the results also highlight the challenges associated with drawing broad conclusions given observed variability in results obtained from different broodstocks and study years.

levels (Ω) decrease (Feely et al. 2004, 2010; Hönisch et al. 2012; Duarte et al. 2013). Coastal zones, in addition to increasing atmospheric CO_2 fluxes, can experience acidification attributable to other mechanisms, including river water inputs (Salisbury et al. 2008), upwelling (Feely et al. 2008), and nutrient loading (Wallace et al. 2014). Accordingly, inshore areas often are exposed to conditions that are not expected to occur in the open ocean for hundreds of years (Cai et al. 2011; Melzner et al. 2013; Carstensen et al. 2018). For example, the Chesapeake Bay estuary has longitudinal and vertical pH and Ω signals that vary seasonally, with both highest and lowest pH (7.31–8.18) and Ω (0.31–3.93) values reported in summer (Cai et al. 2017; Brodeur et al. 2019). Declines in pH and Ω are known to affect molecular, physiological, developmental, and behavioral processes in marine organisms (Seibel and Fabry 2003; Orr et al. 2005; Fabry et al. 2008; Siedlecki et al. 2021). Studying the effects of OA upon marine organisms is complex because the response to different ρCO_2 concentrations may vary with life stage and across species.

Juvenile and adult fish are reported to be more resilient to acidification than calcifying marine invertebrates (Pörtner et al. 2004; Melzner et al. 2009; Hurst et al. 2012; Perry et al. 2015; Davis et al. 2016); however, fish embryos, which are still developing physiological controls, may be vulnerable to OA (Murray et al. 2014; Gobler et al. 2018). Previous studies have suggested species-dependent responses to OA for fish. The early life stages of some fish species are highly sensitive to OA. In a study of two

Ocean acidification (OA), a decrease in ocean pH, is a result of increased anthropogenic carbon dioxide (CO_2) in the atmosphere (Feely et al. 2010; Hönisch et al. 2012; Duarte et al. 2013), which causes dissolved CO_2 (ρCO_2) and bicarbonate ions (HCO_3^-) to increase, while carbonate ions (CO_3^{2-}), pH, and calcium carbonate saturation

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different stocks of Atlantic Cod *Gadus morhua* exposure to high ρCO_2 levels (~1,100 microatmospheres [μatm]) resulted in a doubling of daily mortality rates. This was true for larvae at all feeding levels, even among fish that were fed ad libitum prey densities, where energy resources should have been sufficient for regulation of acid–base processes (Stiasny et al. 2016). Experiments with Inland Silverside *Menidia beryllina* (Baumann et al. 2012; Gobler et al. 2018), Summer Flounder *Paralichthys dentatus* (Chambers et al. 2013), and Atlantic Cod (Stiasny et al. 2016) showed direct reductions in growth and survival when early life stages were exposed to elevated ρCO_2 concentrations (Chambers et al. 2013; Stiasny et al. 2016). In contrast, Walleye Pollock *Gadus chalcogrammus* (Hurst et al. 2012, 2013, 2021) and European Bass *Dicentrarchus labrax* (Crespel et al. 2017) showed little to no sensitivity and no effect of elevated ρCO_2 on growth and survival. Conversely, survival of larval Atlantic Herring *Clupea harengus* exposed to combinations of high ρCO_2 (400 and 900 μatm) and temperature (10°C and 12°C) showed no consistent response (Sswat et al. 2018). A negative effect of ρCO_2 on survival was detected at 12°C, whereas the effect was observed to be positive at 10°C, suggesting that synergetic effects of multiple environmental factors can elicit varying responses to OA in young fish (Sswat et al. 2018). These previous studies highlight the complex response of marine fish to OA but were limited by the scope of ρCO_2 exposure levels, generally focusing on two or three treatments within a narrow range (400, 800, and 1,200 μatm ; Baumann et al. 2012; Chambers et al. 2013; Hurst et al. 2013; Espinel-Velasco et al. 2018). The best practice guide for OA experiments recommends that multiple ρCO_2 levels ranging from preindustrial (<300 μatm) to predicted future levels (>1,200 μatm) are optimal for examining the physiological response of organisms to OA (Riebesell et al. 2010).

An assessment of fish and invertebrate species found on the Northeast U.S. Continental Shelf has identified Black Sea Bass *Centropristis striata* (BSB) as potentially susceptible to climate change (Hare et al. 2016). The BSB is an economically valued species in the mid-Atlantic United States, with a 2018 recreational fisheries harvest of 5.44 million kg (12 million lb) and commercial fisheries landings of 1.45 million kg (3.2 million lb; NOAA Fisheries 2019). Historically, BSB have ranged from Massachusetts to the Gulf of Mexico, with the stock centered in the Middle Atlantic Bight (Roy et al. 2012; Slesinger et al. 2019). More recently, the northern stock of BSB has shifted northward into the Gulf of Maine, with the shift attributed to warming water temperatures (Bell et al. 2015; Hare et al. 2016; Kleisner et al. 2017; McMahan et al. 2020). Adults migrate seasonally from January through July for spawning, moving into coastal estuaries (e.g., Cape Hatteras, Chesapeake Bay, and the Delaware and

Hudson River estuaries; Drohan et al. 2007), where OA levels may be more variable than in the open ocean.

Currently, little is known regarding the effects of elevated ρCO_2 upon BSB, especially during early life stages, when fish may be more sensitive to OA effects (Ishimatsu et al. 2008; Murray et al. 2014). In this preliminary study, we exposed embryos to a range of ρCO_2 levels (182.7–2,252.6 μatm) and measured the proportions of hatched larvae and unhatched viable embryos after 48 h of exposure. Our hypothesis was that ρCO_2 concentrations would be an accurate predictor of hatching success. Larvae were also assessed for vertebral column anomalies (scoliosis and/or lordosis) and were classified as normal or abnormal.

METHODS

Adult male and female BSB broodstock were held at the National Oceanic and Atmospheric Administration (NOAA) Milford Laboratory (Milford, Connecticut) under ambient seawater conditions for Long Island Sound (41°12'43.6788"N, 73°3'7.47"W) during 2012–2013, 2013–2014, and 2014–2015. The BSB broodstock used in 2013 (7 females, 4 males) were held for approximately 375 d, those used in 2014 (10 females, 6 males) were held for 265 d, and those used in 2015 (11 females, 6 males) were held for 630 d. The mean surface pH values \pm SE recorded at 16 stations distributed within Connecticut waters from the eastern to western end of Long Island Sound ($n = 32$ per year) were 7.65 ± 0.04 in 2013, 7.70 ± 0.04 in 2014, and 7.81 ± 0.04 in 2015 (Connecticut Department of Energy and Environmental Protection; Water Quality Portal, <https://www.waterqualitydata.us/portal/#mimeType=csv>).

These values varied seasonally, ranging from 7.59 to 7.71 in 2013, from 7.64 to 7.76 in 2014, and from 7.74 to 7.87 in 2015. Adults were fed squid during conditioning and spawning. Gamete development in fish occurred naturally, and spawning took place in holding tanks during July of each year. Outflow water from the broodstock tank passed through a 500- $\mu\text{m} \times 30.5\text{-cm}$ round screen (placed in a basin) to collect the eggs. To reduce variation in embryonic stage between treatments and across years, all eggs were examined under an inverted microscope to select only those eggs that had reached blastula development (~6 h after fertilization). These embryos were then exposed to ρCO_2 levels for 48 h. Eight experimental trials were conducted during 2013 ($n = 3$), 2014 ($n = 4$), and 2015 ($n = 1$). One trial conducted in 2014 was removed from analysis because hatching was less than 1%, which was highly atypical compared to the results for the other trials. A trial run consisted of exposing embryos to one of three ρCO_2 levels (low, medium, or high), with three experimental beakers for each ρCO_2 level, resulting in nine independent ρCO_2 concentrations. For one trial in

2013, there were only enough blastula-stage embryos for two ρCO_2 levels.

Experiments were conducted in 3-L beakers. Each beaker was placed in a temperature-controlled (mean \pm SD = $20.3 \pm 0.6^\circ\text{C}$) water table. Salinity in each beaker was measured using a Yellow Springs Instruments Model 85 handheld meter at time 0 and at 48 h, with no detectable change in salinity. Artificial seawater was combined with natural seawater (salinity of 27) from Milford Harbor ($41^\circ 12' 43.6788''\text{N}$, $73^\circ 37' 47''\text{W}$) to raise the salinity to 32.32 ± 0.20 . Target ρCO_2 levels represented a range of conditions from preindustrial to projected future levels (182.7 – $2,252.6 \mu\text{atm}$). Mass-flow controllers (Aalborg Instruments and Controls, Orangeburg, New York) delivered a mixture of research-grade CO₂ and compressed air that passed through a CO₂ absorber (Puregas, Broomfield, Colorado). This CO₂/air mixture was delivered by an air stone to each beaker to maintain ρCO_2 levels that had been measured and set prior to the addition of embryos. Each 3-L beaker was stocked with 50 blastula-stage embryos in 2013 and 100 blastula-stage embryos in 2014 and 2015.

Black Sea Bass are known to hatch within 48 h at 20°C (Perry et al. 2007). To quantify hatching success, embryos and larvae were removed from each beaker after 48 h of ρCO_2 exposure and were preserved in 10% buffered formalin for analysis. Fish larvae and embryos that are preserved in 10% formalin are known to remain stable for up to 29 weeks with well-developed features (Klinger and van Den Avyle 1993). Viable embryos were those observed to be alive at sampling, while nonviable embryos sank to the bottom (i.e., were dead) and had no development beyond the blastula stage. Black Sea Bass were examined under a microscope to count hatched larvae and unhatched viable embryos and to assess larvae for vertebral column abnormalities. Each preserved larva was assessed for anomalies by two different individuals for quality control to ensure accuracy and consistency. Hatching success (%) was calculated as the number of BSB larvae divided by the total stocking density $\times 100$. To assess whether increased ρCO_2 levels reduced hatching in BSB, the percentage of unhatched viable embryos was calculated as the number of viable embryos divided by the total stocking density $\times 100$. Newly hatched larvae with poorly differentiated skeletal tissue were scored as abnormal if they showed sublethal vertebral column anomalies, including lordosis (V-shaped dorsal–ventral curvature) or scoliosis (lateral curvature; Roo et al. 2009; Boglione et al. 2013). Vertebral column anomalies (%) were calculated as the number of abnormal larvae divided by the total number of larvae $\times 100$.

Seawater samples collected from each beaker prior to the addition of the BSB embryos and when the experiment

ended (time = 48 h) were analyzed for pH (U.V. Spectrometer; Ocean Optics, Winter Park, Florida) and dissolved inorganic carbon (DIC; Apollo SciTech, Newark, Delaware) immediately upon collection, without the addition of preservatives. The DIC and pH values showed low variation (<0.2 – 1.5%) over the 48 h; therefore, the mean value was reported and was used in CO₂SYS (Pierrot et al. 2006). Certified reference materials, analyzed along with samples, measured within 1 SD for total DIC ($\pm 2.7 \mu\text{mol/kg}$) replicates and pH (± 0.0014 ; seawater scale). This protocol was part of a previous international, interlaboratory DIC comparison exercise that measured within 0.5% of assigned values (Bockmon and Dickson 2015). The DIC and pH values were entered into CO₂SYS (Pierrot et al. 2006) to calculate ρCO_2 (μatm), Ω_{calcite} , and $\Omega_{\text{aragonite}}$ using the following constants: K_1 and K_2 from Lueker et al. (2000), K of hydrogen sulfate from Dickson (1990), and total boron from Lee et al. (2010).

During 2013, experimental conditions for pH and DIC at in situ temperature (20°C) ranged from 7.242 to 8.277 and from 1,642.66 to 2,188.69 $\mu\text{mol/kg}$, respectively (Table 1). The calculated levels of ρCO_2 ranged from 177 to 2,698 μatm , while Ω_{calcite} measured from 5.60 to 0.72. In 2014, pH at in situ temperature ranged from 7.197 to 7.934, while DIC measured from 1,875.79 to 2,208.76 $\mu\text{mol/kg}$. This corresponds to a calculated ρCO_2 level between 480 and 2,942 μatm and Ω_{calcite} between 3.15 and 0.64. Treatments were similar in 2015, with a pH that ranged from 7.445 to 7.979 and DIC values of 1,842.83 to 2,018.86 $\mu\text{mol/kg}$; calculated ρCO_2 was between 423 and 1,641 μatm , and Ω_{calcite} ranged from 3.39 to 1.12.

All statistical analyses were performed with R version 4.1.1 (R Foundation for Statistical Computing, Vienna; www.r-project.org) using robust methods described by Wilcox (2017) and employing R source library version 39 (downloaded from <https://dornsife.usc.edu/labs/rwilcox/software/>). A running interval smoother was used to assess the explanatory power of ρCO_2 concentration in predicting BSB metrics (Wilcox 2017). Smoothers are advantageous over the ordinary least-squares regression or Pearson's product-moment correlation approaches because they are robust to conditions of nonnormality or heteroscedasticity as well as to the presence of outliers in the data. Smoothers also do not assume a linear relationship and have a better fit to nonlinear data while still maintaining a good fit for relationships that are linear or zero. Smoothers are particularly effective over classical regression techniques in threshold responses where abrupt changes in the relationship between the predictor and response variables may occur (Wilcox 2017). Previous studies (Barry et al. 2010; Ries et al. 2010) have shown that the biological metric relationship with ρCO_2 can be zero or linear up to a certain threshold ρCO_2 concentration before changing abruptly in a nonlinear fashion; thus,

TABLE 1. Chemical parameters for carbonate chemistry in blastula-stage Black Sea Bass embryos that were exposed to ρCO_2 levels for 48 h. Dissolved inorganic carbon (DIC; $\mu\text{mol/kg}$) and pH values were entered into CO2SYS (Pierrot et al. 2006) to calculate ρCO_2 (microatmospheres [μatm]) and calcium carbonate saturation (Ω_{calcite}) using the following constants: K_1 and K_2 from Lueker et al. (2000), K of hydrogen sulfate from Dickson (1990), and total boron from Lee et al. (2010). The reported pH value is at 20°C.

Date	ρCO_2 level	Salinity	DIC	pH	ρCO_2	Ω_{calcite}
Jul 13, 2013	Low	32	1,642.66	8.28	177.03	5.60
	Low	32	1,748.84	8.23	213.21	5.43
	Low	32	1,755.51	8.21	222.54	5.28
Jul 13, 2013	Medium	32	1,897.49	7.71	823.56	1.95
	Medium	32	1,989.64	7.73	831.82	2.12
	Medium	32	2,005.07	7.73	832.33	2.15
Jul 13, 2013	High	32	2,097.42	7.39	1,912.30	1.04
	High	32	2,020.73	7.37	1,943.06	0.95
	High	32	2,101.50	7.36	2,074.90	0.96
Jul 20, 2013	Low	32.5	1,819.20	7.85	566.30	2.55
	Low	32.5	1,813.66	7.85	568.56	2.52
	Low	32.5	1,835.64	7.80	646.93	2.29
	Low	32.5	1,830.81	7.81	632.69	2.33
Jul 20, 2013	High	32.5	1,936.69	7.48	1,455.82	1.17
	High	32.5	1,956.76	7.40	1,753.52	0.99
Jul 23, 2013	Low	32.3	1,933.70	7.99	432.93	3.62
	Low	32.3	1,899.99	8.00	415.32	3.63
	Low	32.3	1,866.66	8.01	393.56	3.68
Jul 23, 2013	Medium	32.3	1,993.36	7.78	745.22	2.35
	Medium	32.3	1,946.55	7.76	766.32	2.19
	Medium	32.3	1,960.48	7.76	765.07	2.22
Jul 23, 2013	High	32.3	2,188.69	7.45	1,753.36	1.23
	High	32.3	2,094.08	7.24	2,697.91	0.72
	High	32.3	2,083.33	7.42	1,797.63	1.09
Jul 3, 2014	Low	32	1,878.20	7.84	610.12	2.52
	Low	32	1,888.66	7.82	638.47	2.44
	Low	32	1,875.79	7.84	600.61	2.55
Jul 3, 2014	Medium	32	2,008.27	7.48	1,508.67	1.20
	Medium	32	2,022.98	7.39	1,886.44	0.97
	Medium	32	2,008.38	7.44	1,652.44	1.10
Jul 3, 2014	High	32	2,072.09	7.21	2,881.65	0.66
	High	32	2,065.03	7.25	2,598.90	0.73
	High	32	2,064.29	7.20	2,941.96	0.64
Jul 5, 2014	Low	32.5	1,876.28	7.93	486.17	3.10
	Low	32.5	1,876.62	7.93	480.12	3.13
	Low	32.5	1,893.18	7.93	486.37	3.15
Jul 5, 2014	Medium	32.5	2,088.18	7.44	1,700.85	1.16
	Medium	32.5	2,094.54	7.43	1,762.47	1.13
	Medium	32.5	2,086.13	7.45	1,689.08	1.17
Jul 5, 2014	High	32.5	2,208.76	7.39	2,039.22	1.08
	High	32.5	2,177.54	7.29	2,540.44	0.84
	High	32.5	2,184.87	7.23	2,911.53	0.73
Jul 12, 2014	Low	32.5	1,907.94	7.90	529.58	2.96
	Low	32.5	1,911.73	7.89	544.97	2.90
	Low	32.5	1,901.66	7.88	549.24	2.85
Jul 12, 2014	Medium	32.5	2,019.44	7.48	1,530.29	1.21
	Medium	32.5	2,028.83	7.49	1,482.71	1.26
	Medium	32.5	2,024.52	7.56	1,254.54	1.48

TABLE 1. Continued.

Date	ρCO_2 level	Salinity	DIC	pH	ρCO_2	Ω_{calcite}
Jul 12, 2014	High	32.5	2,105.50	7.35	2,135.91	0.94
	High	32.5	2,076.86	7.40	1,869.73	1.04
	High	32.5	2,094.55	7.30	2,358.74	0.84
Jul 10, 2015	Low	32	1,885.25	7.97	440.67	3.39
	Low	32	1,846.61	7.97	431.42	3.32
	Low	32	1,842.83	7.98	423.35	3.37
Jul 10, 2015	Medium	32	1,979.09	7.68	938.42	1.86
	Medium	32	1,957.34	7.64	1,025.27	1.67
	Medium	32	1,972.94	7.63	1,047.24	1.66
Jul 10, 2015	High	32	2,016.87	7.45	1,631.18	1.12
	High	32	2,018.86	7.45	1,639.06	1.12
	High	32	2,016.43	7.45	1,641.00	1.12

we have employed smoothers as the approach most likely to detect a broad range of potential relationships between the predictor and response variables. The running interval smoother applied here enabled the use of a robust analog of the sample mean—the 20% trimmed mean—as a measure of location to calculate a series of y -values (fish metric) moving across the observed predictor x -values (ρCO_2 concentration). The 20% trimmed mean was chosen as the measure of location in the response data because this metric has been shown to be much more robust to the presence of outliers than the standard sample mean (Wilcox 2017). The running interval smoother uses a robust analog of the SD—the median absolute deviation—to determine which x -values are considered close to a given point for the purpose of calculating the 20% trimmed means, and the span was set to $f=1$. Each beaker was considered a replicate, with beaker-specific measurements of ρCO_2 concentration paired to all biological metrics. Running interval smooths were generated for each biological metric (hatching success, unhatched viable embryos, vertebral column anomalies) for data collected within each individual year (2013: $n=24$; 2014: $n=27$; 2015: $n=9$). Data were also pooled across all 3 years to allow for an examination of broader trends in the data ($n=60$). Although the running interval smoother does not provide a test of significance or generate a predictive equation, it does provide a measure of explanatory power, and its flexibility makes it useful as an exploratory data analysis tool. Smooths with an explanatory power greater than zero were selected for further analysis and examined visually for evidence of response thresholds. If the smooth suggested a consistent trend across the range of observed ρCO_2 concentrations, the robust Theil–Sen regression estimator was used to generate a predictive equation, and the significance of the estimator was tested using a percentile bootstrap method, which does not assume normality or

equality of variance in the data (Wilcox 2017). If the smooth suggested a threshold response in the data, we separated the data into two groups based on the threshold ρCO_2 concentration, and those groups were compared using 20% trimmed means as the measure of location and a percentile bootstrap method for hypothesis testing (Wilcox 2017).

RESULTS

Overall, hatching success showed no relationship with ρCO_2 concentration when all years were combined or for the 2013 and 2015 individual year data sets (Table 2). During 2014, ρCO_2 concentration explained 13% of the variation in hatching success. Regression analysis suggested a negative relationship between hatching success and ρCO_2 concentration (slope = -4.9×10^{-5} ; intercept = 0.53), but the slope was not statistically significant ($P=0.073$). The percentage of hatched larvae ranged from 6% to 63% in 2013, from 16% to 70% in 2014, and from 36% to 95% in 2015 (Figure 1).

When all years were pooled, ρCO_2 explained 39% of the variation in the percentage of unhatched viable embryos within 48 h (Table 2) and the smooth was curvilinear in shape (Figure 2). Regression analysis suggested a positive relationship between ρCO_2 concentration and the percentage of unhatched viable embryos (slope = 4.9×10^{-2} ; intercept = 6.2×10^{-5}), and the slope was statistically significant ($P=0.03$). The explanatory power of the Theil–Sen regression estimator (16%) was less than half that of the smooth (39%), likely due to the curvature in the data. In 2013, the ρCO_2 concentration explained 38% of the variation in the percentage of unhatched embryos, and 0–20% of the embryos did not hatch (Figure 2). Regression analysis suggested a positive relationship between ρCO_2 concentration and the percentage of unhatched viable

TABLE 2. Explanatory power of ρCO_2 concentration in predicting Black Sea Bass hatching success, percentage of unhatched viable embryos, and frequency of vertebral column anomalies based on a running interval smoother.

Metric	Data set	<i>n</i>	Explanatory power
Hatching success	2013	24	0.00
	2014	27	0.13
	2015	9	0.00
	All years	60	0.00
Unhatched viable embryos	2013	24	0.38
	2014	27	0.00
	2015	9	0.00
	All years	60	0.39
Frequency of anomalies	2013	24	0.00
	2014	27	0.40
	2015	9	0.12
	All years	60	0.38

embryos (slope = 1.5×10^{-5} ; intercept = 3.6×10^{-2}), but the slope was not statistically significant ($P=0.33$). In 2014 and 2015, there was no relationship between ρCO_2 concentration and the percentage of unhatched viable embryos. Approximately 11–55% of the embryos did not hatch in 2014, while 0–8% did not hatch in 2015.

The frequency of vertebral column anomalies showed no trend when data were pooled across years (explanatory power = 0; Table 2). Results across individual years were mixed. The ρCO_2 concentration explained 40% of the variation in vertebral column abnormalities in 2013, and a visualization of the smooth suggested a declining trend at low ρCO_2 but an increasing trend above 1,000 μatm (Figure 3). Regression analysis over the full range of ρCO_2 concentrations yielded a positive overall slope, although it was not significant (slope = 8.0×10^{-5} ; intercept = 0.41; $P=0.32$). Data were also separated into two groups based on a 1,000- μatm threshold, but statistical comparison of these groups was not significant ($P=0.10$). The ρCO_2 concentration explained 13% of the variability in vertebral column abnormalities in 2014, but the smooth suggested a declining trend. Regression analysis yielded a negative slope (slope = -1.34×10^{-5} ; intercept = 0.46), but the slope was not statistically significant ($P=0.62$). The concentration of ρCO_2 explained 38% of the variability in vertebral column abnormalities in 2015, and the smooth suggested a consistent increase across ρCO_2 concentrations. Regression analysis yielded a positive but nonsignificant slope (slope = 1.6×10^{-4} ; intercept = 0.55; $P=0.15$). The frequency of vertebral column anomalies ranged from 22% to 90% in

2013, from 8% to 80% in 2014, and from 46% to 86% in 2015 (Figure 3).

DISCUSSION

Absence of an organ system and limited homeostatic regulation in embryos suggest that developing eggs may be more vulnerable to OA than other life stages. Hatching often presents a critical bottleneck in the life history of marine fish (Köster et al. 2003; Houde 2008; Dahlke et al. 2020a). Reduced hatching in response to high ρCO_2 has been observed in some marine fish, including Senegalese Sole *Solea senegalensis* (Pigmental et al. 2014; Faria et al. 2017), White Seabream *Diplodus sargus*, and Meagre *Argyrosomus regius*, as well as crustaceans, including Florida stone crab *Menippe mercenaria* (Gravinese 2018), Antarctic krill *Euphausia superba* (Kawaguchi et al. 2013), and the copepod *Calanus finmarchicus* (Mayor et al. 2007).

We observed no relationship between ρCO_2 and hatching during our 2013 and 2015 trials, and while the running interval smoother suggested a slight declining trend in hatching success with increasing ρCO_2 in 2014 (explanatory power = 13%), subsequent regression analysis was not statistically significant ($P=0.07$). These results are similar to those of other studies (Atlantic Herring *Clupea harengus*: Franke and Clemmesen 2011; Atlantic Cod: Frommel et al. 2013; Yellowtail Jack *Seriola lalandi*: Munday et al. 2016), which found that embryos and larvae can tolerate a variety of ρCO_2 levels that are well above those predicted for the next century. For example, Yellowtail Jack from 12 h postfertilization embryos to 3 d posthatch larvae exhibited tolerance to ρCO_2 levels as high as 1,700 μatm (Munday et al. 2016). Duration of the embryonic and larval periods may play a role in the CO_2 sensitivity of early stage marine fish (Baumann 2019). Species with quickly developing embryos and larvae, like the BSB, may be less susceptible to effects of elevated ρCO_2 than fish with longer-duration early life stages (Murray et al. 2019), which might explain why an overall relationship of ρCO_2 with hatching success was not consistently observed for all 3 years combined. Furthermore, BSB are migratory fish that move offshore and south in the fall, returning north and inshore to estuaries and bays in the spring. These movements expose them to a variety of ρCO_2 levels, and for this reason, BSB may tolerate a broader range of environmental conditions than do open-ocean fish species (Baumann 2019).

Exposure to ρCO_2 may affect time to hatch for BSB embryos. The ρCO_2 concentration explained 39% of the variability in the percentage of viable unhatched embryos that we observed at 48 h postfertilization when data were combined across all 3 years; there was a significant increasing trend with increased ρCO_2 and the response was clearly nonlinear. Failure to hatch within this time

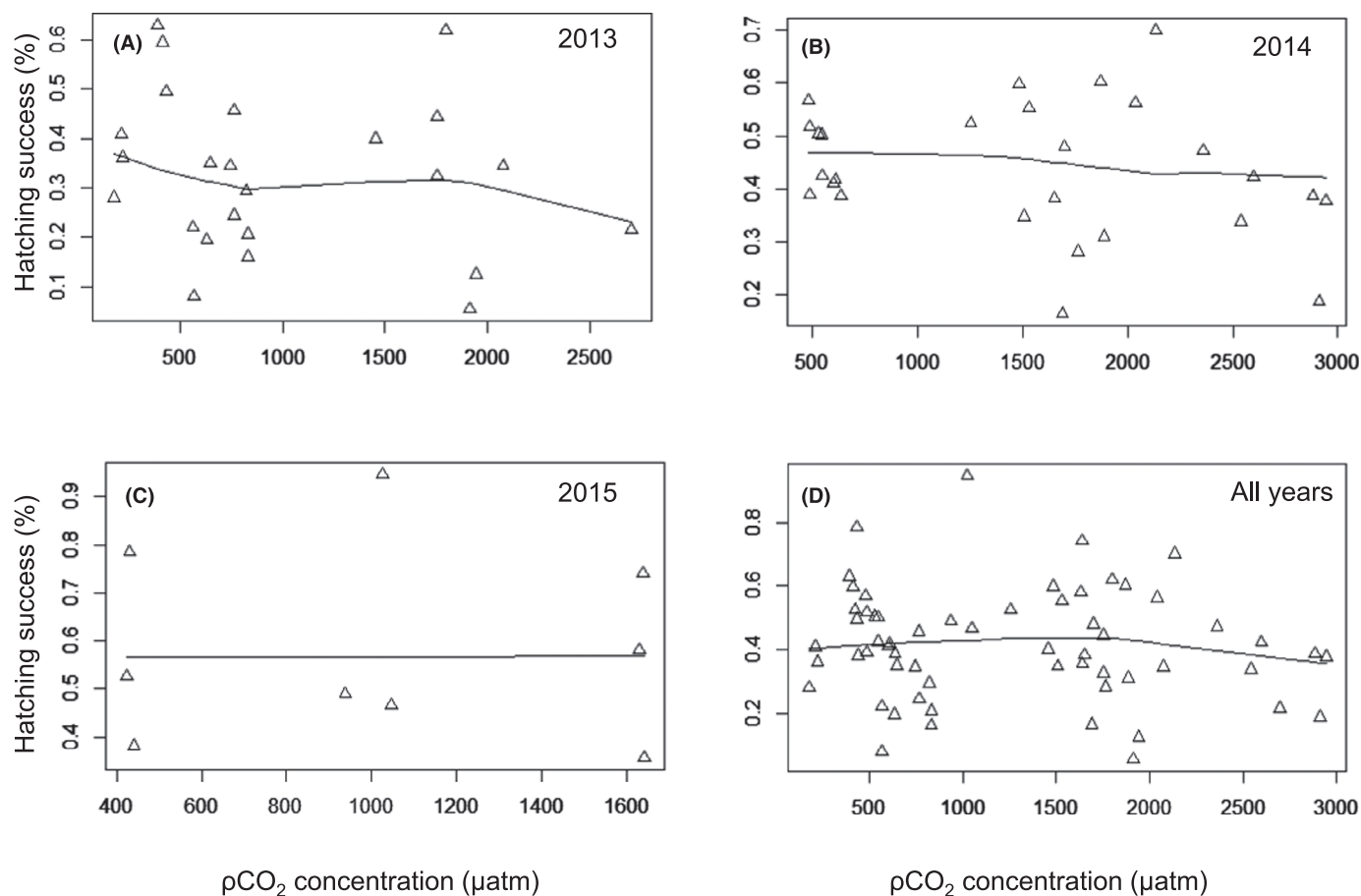


FIGURE 1. Running interval smooths for predicting Black Sea Bass hatching success (%) from the ρCO_2 concentration (microatmospheres [μatm]). Smooths were generated for data from each individual year and all years pooled. Hatching success was calculated as the total number of larvae divided by the total stocking density of fertilized eggs $\times 100$.

frame may indicate a disruption in normal hatching processes, since BSB eggs typically hatch within 48 h at temperatures of 19–20°C (Perry et al. 2007; Watanabe et al. 2021). Although embryos of many marine fish demonstrate tolerance to elevations in ρCO_2 (Munday et al. 2016; Rodrigues-Dominguez et al. 2018), this ability may relate to how quickly their ion channels can respond to varying ρCO_2 levels (Melzner et al. 2009). Embryos do not have gills to maintain acid–base balance; therefore, protons and ions play a critical role in responding to increased ρCO_2 conditions (Melzner et al. 2009). Under elevated ρCO_2 , the ability of embryos to exchange gas ions via transport by channels and pumps may be impaired, which could affect time to hatch (Melzner et al. 2009; Carter et al. 2013; Pan et al. 2015), and this mechanism may account for the increase in unhatched viable embryos with elevated ρCO_2 . A possible disruption to hatching enzyme levels might have also contributed to the delay in time to hatch for BSB embryos. Levels of hatching enzymes are known to be dependent on the pH of the

water (Swain et al. 2020) and may have been affected by elevations in ρCO_2 . Although it was beyond the scope of this study to explore all of the possible mechanisms that could relate to variation in hatching, our trials suggest that time to hatch in BSB may be prolonged at high ρCO_2 concentrations. Further studies are needed to better understand intrinsic factors that may influence the response to ρCO_2 , including fluctuations in egg quality, ion channel and pump response times, sensitivity of hatching enzymes to pH, and the influence of parental lineage.

We found that ρCO_2 concentration was a poor predictor of the frequency of vertebral column anomalies when data were pooled across years, and we obtained mixed results when data were examined by individual year. The smooth generated for the 2013 data set had good explanatory power (40%) and suggested the presence of a possible threshold around 1,000 μatm , with different responses on either side of that point, but statistical comparison of the data above and below 1,000 μatm produced nonsignificant results. A linear regression of the data yielded a positive

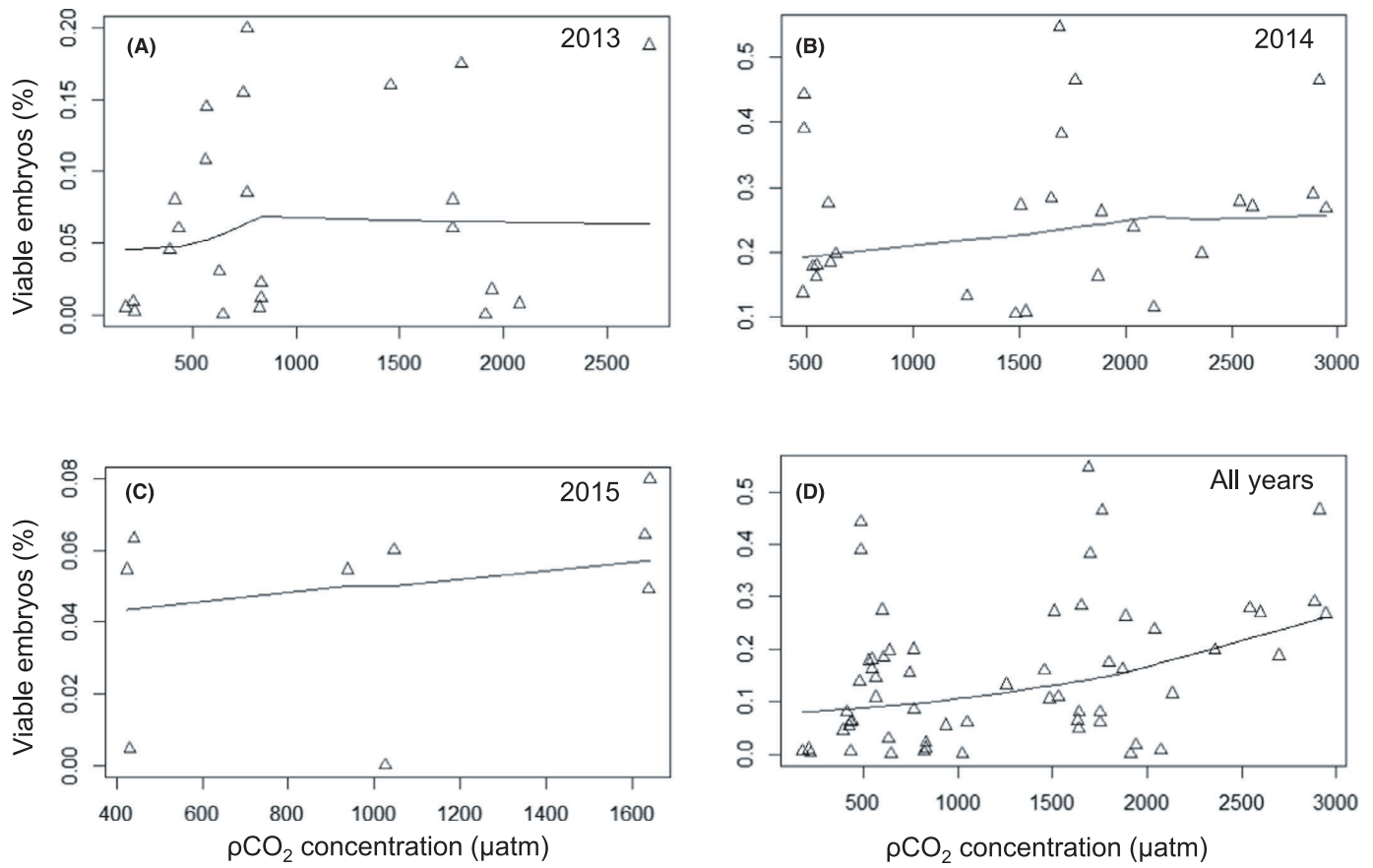


FIGURE 2. Running interval smooths for predicting the percentage of unhatched viable Black Sea Bass embryos at 48 h from the ρCO_2 concentration (microatmospheres [μatm]). Smooths were generated for data from each individual year and all years pooled. The percentage of unhatched viable embryos was calculated as the total number of live embryos that had not hatched after 48 h divided by the total stocking density \times 100.

slope, but the slope was not significant and had poor explanatory power (8%) relative to the smooth. Results from 2014 were not significant, and the smooth suggested a slight decreasing trend in vertebral column anomalies with increasing ρCO_2 . In 2015, the smooth generated had good explanatory power (38%) and the overall trend was increasing vertebral column anomalies with increased ρCO_2 , but the data set was small ($n=9$) and the slope of the subsequent regression line was not significant.

Under culture conditions, the presence of vertebral column anomalies can be high in marine fish larvae under ambient ρCO_2 . For example, in Red Porgy *Pagrus pagrus*, up to 50% of larvae within a cohort can be affected (Roo et al. 2009), while Atlantic Salmon *Salmo salar* have been shown to experience wide variation (7–94%) in the prevalence of anomalies (Grini et al. 2011). Evidence of vertebral column anomalies under increased ρCO_2 has also been observed in adult fish from shallow CO_2 vents (Mirasole et al. 2017, 2021) and at the early life stages (egg to larvae) in Senegalese Sole (Pimentel et al. 2014) and

Gilthead Bream *Sparus auratus* (Pimentel et al. 2016). Many factors can contribute to abnormalities, as larvae are sensitive to environmental and handling conditions during early osteological development. Alterations in vertebral column development can result from suboptimal environmental conditions (Incardona et al. 2004; Boglione et al. 2013) and nutritional deficiencies (Hamre et al. 2013; Rønnestad et al. 2013) and can be influenced by hereditary factors (Castro et al. 2008; Boglione et al. 2013). The ecophysiology of larval fish may be compromised by vertebral column anomalies (Pimentel et al. 2014), which can (1) affect their ability to position themselves in the water column, (2) impede swimming and foraging, and (3) interfere with predator–prey interactions (Powell et al. 2009). Functional transitions, such as hatching and metamorphosis, are critical periods during development in which physical defects may result in mortality or reduce the competency of larval or juvenile fish (Dahlke et al. 2016, 2020b). Future studies should focus on long-term effects of vertebral anomalies.

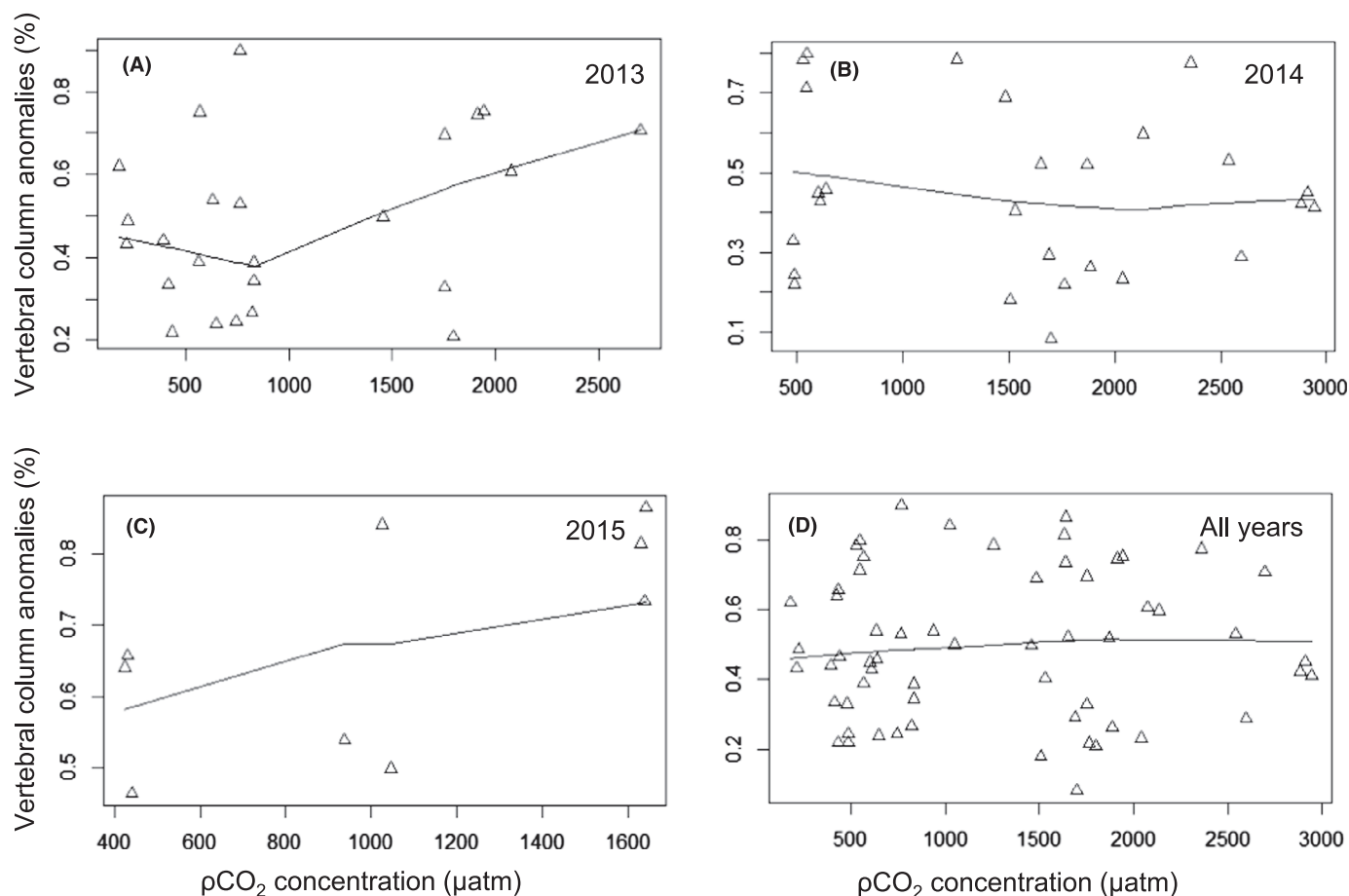


FIGURE 3. Running interval smooths for predicting the percentage of Black Sea Bass larvae with vertebral column anomalies (%) from the ρCO_2 concentration (microatmospheres [μatm]). Smooths were generated for data from each individual year and all years pooled. The frequency of vertebral column anomalies was calculated as the number of larvae that had lordosis and/or scoliosis divided by the total number of larvae $\times 100$.

Although our preliminary results found variability in the response of BSB embryos to ρCO_2 , the early life stages of BSB appear generally resilient to the ρCO_2 levels that are predicted to occur over the next 100 years (800–1,000 μatm). We found no apparent effects of ρCO_2 upon hatching, but we did observe a modest increase in time to hatch and mixed results in vertebral column anomalies with rising levels of ρCO_2 exposure during some trials. Observed effects generally were nonlinear, with curvature observed in the response of time to hatch and one potential threshold observed in 2013 for vertebral column anomalies. Interannual variability in the response of BSB embryos to ρCO_2 exposure, however, highlights the challenges of documenting robust predictive relationships between ρCO_2 concentration and biological metrics. Variability in egg quality between broodstocks has been reported in the literature and may have contributed to differences in the embryo responses we observed across study years. Further research should focus on critical life history stages that may be key in determining developmental

resilience to a range of environmental conditions and should be conducted concurrently with exposure to other climate change variables (i.e., temperature).

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