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Effects of elevated CO_2 in the early life stages of summer flounder, *Paralichthys dentatus*, and potential consequences of ocean acidification

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Abstract. The limited available evidence about effects on marine fishes of high CO_2 and associated acidification of oceans suggests that effects will differ across species, be subtle, and may interact with other stressors. This report is on the responses of an array of early life history features of summer flounder (*Paralichthys dentatus*), an ecologically and economically important flatfish of the inshore and nearshore waters of the Mid-Atlantic Bight (USA), to experimental manipulation of CO_2 levels. Relative survival of summer flounder embryos in local ambient conditions (775 µatm pCO_2, 7.8 pH) was reduced to 48 % when maintained at intermediate experimental conditions (1808 µatm pCO_2, 7.5 pH), and to 16 % when maintained at the most elevated CO_2 treatment (4714 ppm pCO_2, 7.1 pH). This pattern of reduced survival of embryos at high-CO_2 levels at constant temperature was consistent among offspring of three females used as experimental subjects. No reduction in survival with CO_2 was observed for larvae during the first four weeks of larval life (experiment ended at 28 d post-hatching (dph) when larvae were initiating metamorphosis). Estimates of sizes, shapes, and developmental status of larvae based on images of live larvae showed larvae were initially longer and faster growing when reared at intermediate- and high-CO_2 levels. This pattern of longer larvae – but with less energy reserves at hatching – was expressed through the first half of the larval period (14 dph). Larvae from the highest-CO_2 conditions initiated metamorphosis at earlier ages and smaller sizes than those from intermediate- and ambient-CO_2 conditions. Tissue damage was evident in larvae as early as 7 dph from both elevated-CO_2 levels. Damage included dilation of liver sinusoids and veins, focal hyperplasia on the epithelium, and separation of the trunk muscle bundles. Cranio-facial features changed with CO_2 levels in an age-dependent manner. Skeletal elements of larvae from ambient-CO_2 environments were comparable or smaller than those from elevated-CO_2 environments when younger (7 and 14 dph) but were larger at developmental stage at older ages (21 to 28 dph), a result consistent with the accelerated size-development trajectory of larvae at higher-CO_2 environments based on analysis of external features. The degree of alterations in the survival, growth, and development of early life stages of summer flounder due to elevated-CO_2 levels suggests that this species will be increasingly challenged by future ocean acidification. Further experimental studies on marine fishes and comparative analyses among those studies are warranted in order to identify the species, life stages, ecologies, and responses likely to be most sensitive to increased levels of CO_2 and acidity in future ocean waters. A strategy is proposed for achieving these goals.

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1 Introduction

Ocean acidification (OA) results from the absorption of atmospheric CO_2 by ocean water. OA is projected to increase for at least the next several centuries, and is likely to have pervasive effects at individual, population, and ecosystem levels (Doney et al., 2009; Fabry et al., 2008). Various scenarios from models of climate change predict the levels of CO_2 to more than double from current conditions by the end of this century. These models are typically configured for open-ocean biogeochemistry where current CO_2 concentrations are approximately 390 ppm pCO_2. Model scenarios predict CO_2 to increase to over 750 ppm by 2100 and to increase over fivefold by 2300 (Caldeira and Wickett, 2003; IPCC, 2007; Feely et al., 2009; Meinshausen et al., 2011). Along with the predicted increases in atmospheric and oceanic CO_2 over these time periods, surface ocean water pH is expected to decrease by 0.25 to up to 0.7 units. The current and predicted levels of CO_2 and acidity of seawater for nearshore, estuarine, and higher-latitude habitats are expected to be even greater and substantially more variable than those for the open ocean (Gruber et al., 2012; Duarte et al., 2013; Johnson et al., 2013). Importantly, these inshore temperate to boreal regions of higher and more variable CO_2 levels are also where a large fraction of the commercially extractable living marine resources reside.

Research on OA effects in marine fishes is in its infancy, with the large majority of publications having appeared in the last five years. This small but growing body of studies on the OA effects in marine fishes is impressive in the diversity of study species, their resident ecosystems, and the broad array of response variables evaluated. Reports to date include those on tropical reef fishes (Munday et al., 2009b), a pantropical rachycentrid (Bignami et al., 2013), a temperate sciaenid (Checkley et al., 2009), an estuarine atherinopsid (Baumann et al., 2012), a gadid from the NE Pacific shelf (Hurst et al., 2013), and a clupeid (Franke and Clemmeson, 2011) and gadid (Frommel et al., 2012, 2013) from the low-salinity Baltic Sea. The responses evaluated for evidence of CO_2 effects include activity of sperm (Frommel et al., 2010) and the survival (Baumann et al., 2012), growth (Hurst et al., 2013), condition (Franke and Clemmesen, 2011), tissue and organ development (Frommel et al., 2012), otolith morphometry (Checkley et al., 2009; Bignami et al., 2013), olfactory capabilities (Dixson et al., 2010), sidedness (Domenici et al., 2011), homing ability (Munday et al., 2009a), and predator avoidance (Ferrari et al., 2011) of larvae and young juveniles.

If any general pattern can be drawn from published studies, it is that the effects of OA in marine fishes can be expected to (i) vary among species, life stages, and individuals; (ii) be more pronounced in the youngest life stages which have not yet achieved homeostasis with respect to internal acid–base balances; (iii) be subtle yet potentially chronic; and (iv) possibly interact with other stressors. These expectations will be challenging to test. They will demand a carefully planned, strategic approach in order to efficiently create a basis from which predictive generalizations can be drawn.

Few studies have addressed OA effects in marine fishes of direct economic importance. Exceptions include studies on Baltic cod, *Gadus morhua* (Frommel et al., 2010, 2012, 2013); Baltic herring, *Clupea harengus* (Franke and Clemmesen, 2011); and walleye pollock, *Theragra chalcogramma* (Hurst et al., 2013). None has evaluated and reported effects of OA in fishes from the western North Atlantic. Without such information, our ability to predict responses of fishes and other living marine resources to a changing climate in general and elevated levels of CO_2 in particular is severely impaired.

This study is part of a larger research effort funded by the US National Oceanic and Atmospheric Administration's (NOAA) Ocean Acidification Program and is the first of several studies at our laboratory designed to begin filling this information gap. These studies use fish species that are of economic and ecological importance to the mid-Atlantic region of the USA. The objective of this study was to identify and quantify the effects of elevated levels of CO_2 on the early life stages (ELS) of summer flounder, *Paralichthys dentatus*, an ecologically and commercially important paralichthyid flatfish common to inshore waters of this region.

For this study, the ELS encompass the embryonic stage (fertilization to hatching) and most of the larval stage (from hatching until notochord flexion, which is concurrent with the initiation of metamorphosis into the juvenile morphology). The ELS have been shown to be more susceptible to toxic substances and stressors in general than juveniles and adults (Woltering, 1984). They are likely to be the life stages least capable of regulating internal acid–base balances, and therefore most likely to be at risk to the effects of increased acidity associated with elevated-CO_2 concentrations in their environment. Importantly, the vast majority of mortality in marine fish populations occurs during these ELS, this is when year-class strength is determined, and these stages are often key to population connectedness.

General approach

The experiment reported here is an example of the first step in a three-step sequence of experiments. It is a one-way factorial design with CO_2 concentration as the treatment. The range of CO_2 concentrations used is intentionally broad and spans from our lowest level, which represents current concentrations in local, inshore seawater (New Jersey, USA), to an intermediate level reflecting a two to threefold increase above our low-CO_2 level, to a high-CO_2 level intended as an extreme condition. If no ELS response can be elicited at the highest-CO_2 condition, then a robust conclusion could be drawn regarding the resiliency of the test species to CO_2 challenges. The step-two experiments are two-way factorial designs with CO_2 concentration crossed with a second treatment (e.g., temperature, dissolved oxygen, or contaminants)

that may act as a costressor and potentially interact with CO_2 in its effects on the test organism. The ranges of treatments are again broad (e.g., Candelmo et al., unpublished data). Step-three experiments are also two-way factorial designs ($CO_2 \times$ costressor) but with the range of treatments restricted to those slightly above and below the treatment combinations that elicited the steepest gradient in the response variable(s) in step-two designs. Results from this experimental progression should identify potential responses due to treatment(s), estimates of their magnitudes, and define the functional form of the relationships between responses and CO_2, with and without costressors. The ELS responses measured on the test species in these experiments are intended to cover a wide array of lethal and sublethal impacts. This protocol should also provide guidance regarding the technical merit of candidate responses being considered in other OA studies.

2 Methods

2.1 Species studied

Summer flounder is an ecologically and economically important member of the mid-Atlantic marine and estuarine ecosystem. Adults inhabit continental shelf waters throughout the year, with many ingressing to bays during the warmer months (April through September). Summer flounder become piscivorous as young-of-the-year juveniles and are important predators in this ecosystem thereafter.

Summer flounder spawns buoyant eggs in continental shelf water in the autumn, with peak spawning occurring from September to December (Packer et al., 1999). Spawning occurs as autumn water temperatures are decreasing (between 22 to 14 °C), and embryos and larvae inhabit water of 22 to 8 °C or colder depending on spawning time and latitude. Embryos hatch within 2 to 7 days and larvae settle between 5 and 15 weeks post-hatching, again depending on spawning time and latitude (Able and Kaiser, 1994; Packer et al., 1999). The pelagic waters occupied by summer flounder ELS are spatially and seasonally variable, especially in the autumn, but they are less variable than nearby estuarine habitats with respect to high-frequency temporal changes in CO_2 levels.

2.2 CO_2 experiment implementation

Controlled levels of CO_2 were supplied to the summer flounder ELS by a large-scale, flow-through experimental system designed for multivariable OA experiments. The system consists of a pretreatment stage wherein the source seawater (Sandy Hook Bay, New Jersey) is filtered to 0.35 µm and sterilized with ultraviolet radiation. During this experiment the mean (±SD) pCO_2 (µatm) and pH of ambient seawater supplied to our lab were 1671 ± 197 and 7.40 ± 0.03, respectively. For the experiment, the local ambient seawater pCO_2 was reduced by stripping CO_2 from air (Twin Tower model CAS2-11) and then diffusing that air into the filtered and sterilized seawater. The experimental levels for pCO_2 and pH were set by regulated addition of CO_2 gas to the treated seawater. The CO_2 levels used in this study – low ("ambient"), intermediate, and high – were set using a two-stage regulator with a bubble counter (Milwaukee Instruments MA957 CO_2 regulator) on a CO_2 cylinder (Airgas, bone-dry high purity). The regulated gas was diffused into system water to increase pCO_2 (lower pH) to the preselected levels using three 30 L CO_2 gas–seawater exchange columns. These columns were 10 cm-diameter, 3.5 m-high cylinders (PVC pipe). The columns used counter-current gas exchange wherein the system water enters at the top of each of the three columns and exits at the bottom, while CO_2 enters each column at the bottom and exits at the top, flowing counter to the water current. Water was supplied from these CO_2 column at a rate of 66 mL per minute to each of nine flow-through ELS rearing containers which were placed in a constant-temperature water bath.

Each pH treatment level was measured at multiple frequencies and by several methods. Daily pH measurements were made via the spectrophotometric method (Dickson et al., 2007) (Cary 300 BIO UV Visible Spectrophotometer). Four pH measurements were made per day via benchtop electrode (Hanna HI113 pH meter, Orion HI1043 electrode). Continuous pH measurements were logged by inline Durafet pH sensors (Durafet II, Honeywell UDA analyzer). Water chemistry and treatment validation were based on water drawn from the outflows of each CO_2–seawater exchange column. Comparison of the water chemistry from samples taken from the base of these exchange columns with samples of water from the fish-rearing containers showed no difference between the two. Dissolved inorganic carbon (DIC) samples were taken daily in order to determine pCO_2 levels. Samples were measured on an UIC CM5230 coulometer following methods of Dickson et al. (2007). Measurements for DIC, pH, salinity, and temperature were used to calculate pCO_2, bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), alkalinity, and the saturation state of aragonite and calcite. These calculations were done using the seacarb library in R (Lavigne and Gattuso, 2011). The experiment-wide mean (±SD) pCO_2 (µatm) and pH for the three treatment levels (low, intermediate, and high) were 775 ± 42 and 7.81 ± 0.02; 1808 ± 362 and 7.47 ± 0.10; and 4714 ± 557 and 7.06 ± 0.07, respectively. Observed and derived water chemistry parameter estimates are provided in Supplement Table S1.

2.3 Husbandry and experimental protocols

Adult summer flounder were collected from estuaries, bays, and inshore coastal waters of the inner New York Bight (New York and New Jersey) during June through September, returned to the NOAA Howard Laboratory, and acclimated to captivity. Adults were placed in round tanks (either 1.83 or 2.44 m diameter and 1334 or 4271 L volume) at comparable fish densities, supplied with local seawater from Sandy Hook

Bay with salinity of 20 to 26 (practical salinity units, PSU) and pH of 7.46 to 7.63, and maintained in captivity for 3 to 18 months before spawning. Adults were fed thawed frozen forage fishes three to seven days per week, depending on season, and held in a seasonal light and temperature regime that was manipulated to simulate approaching autumn and therefore spawning conditions. Males ripened spontaneously and females were induced to hydrate oocytes by daily IM injections of luteinizing-hormone-releasing hormone analog at 2 mg per kg wet weight until the size of the ovary indicated that egg maturation was imminent (Berlinsky et al., 1997).

Upon ripening, eggs were stripped from each of three females into dry 6 L plastic pans. Each female's eggs were then mixed with milt pooled from three to five different males. Gametes were flooded with ultraviolet-sterilized, 0.5 µm filtered seawater (salinity elevated to 34 PSU by sea salts) to activate the sperm and initiate fertilization. Embryos were gently aerated in 2 L separatory funnels for 2 h until the first water change, and unfertilized eggs and dead embryos were separated from live embryos by buoyancy. Live embryos from each spawned female (hereafter "sibgroups") were divided into two subsets, with one subset counted into 3 groups of 100 embryos. Each 100-count group was transferred to a mesh basket which was floated in 16 L containers that were plumbed to receive one of three concentrations of CO_2-infused seawater in the experimental system. Incubation baskets in this embryo subexperiment were monitored daily for dead embryos and hatched larvae. Subsets of hatched larvae per basket (i.e., nine CO_2 × sibgroup combinations) were anesthetized (tricaine methanesulfonate) and photographed live in lateral perspective at 25 × magnification with a Zeiss Axiocam HRc color digital camera mounted on a Wild M8 dissecting microscope for later image analyses ($n = 10$), preserved for histopathology ($n = 35$), or preserved as archived samples for other assays.

The other subset of embryos from each sibgroup was maintained until hatching in 34 PSU seawater in static, aerated incubators with twice-daily water change and decanting of dead embryos. At one day post-peak-hatch (dph), nine groups of 500 larvae were used to stock the larval subexperiment (3 CO_2 levels × 3 replicates). Larvae for this experiment were drawn from a pool of parental sources (2 females, 7 males), and assigned to one of nine 16 L polycarbonate plastic larval-rearing containers wrapped in black tarp. These containers were plumbed for flow-through with water volume and residence time of 12 L and 3 h, respectively, and were the same containers that housed the baskets of embryos.

For the first three weeks of the larval subexperiment, larvae were fed rotifers (*Brachionus plicatilis*) enriched with *Nannochloropsis* (Rotigrow) and at densities (0.5 to 20 rotifers per mL) and frequencies (one or two times per day) that increased with larval size. Advanced larvae (∼ 21 dph) were also fed second instar *Artemia* enriched with DHA (Selco) at densities (0.5 to 5 nauplii per mL) and frequencies (one or two times per day) that increased with larval size. Larvae were monitored daily and dead larvae were removed until termination of the experiment at 28 dph. Tanks were siphoned weekly to remove accumulated debris. All embryos and larvae were maintained at 19.5 °C and under a 12 : 12 light : dark photoperiod regime throughout the study.

Subsets of larvae per treatment replicate were sampled weekly through 28 dph. One set of larvae ($n = 10$ per replicate) was anesthetized, photographed live in lateral perspective at 6, 12, or 25 × magnification as described above for hatchlings, and preserved as archived samples. An additional set of larvae ($n = 25$ to 35 per replicate) was collected and preserved for histological analysis of tissues and organs.

2.4 Response variables

It was assumed that the proximate cause of effects in ELS responses was due to CO_2 levels disrupting the acid–base equilibria at the cellular level, and that this disruption was likely to be variously expressed at higher levels of biological organization within the individual (e.g., physiological, anatomical, behavioral, and life history responses). To some degree, these different types of response variables are interrelated and represent different manifestations of the underlying effects of elevated CO_2. The response variables were expected to have different sensitivities to CO_2 and to respond on different timescales but all have the potential to be correlated with individual fitness through their direct relationship with the condition, capability, and likelihood of survival of these individuals during the ELS.

The primary response variables in the embryo subexperiment were survival to hatching, and the size and shape of hatchlings. Response variables in the larval subexperiment were survival, size, shape, developmental markers, and key tissues and organs of feeding larval during the major portion of the larval period.

2.4.1 Survival

The numbers of embryos surviving to hatching were based on daily inspection of the embryos in the CO_2-exposure incubation baskets. Hatch-frequency data were summed and converted to proportions of the start group of 100 per incubation basket that survived to hatching. Survival of larvae was based on the numbers initially allocated to each container ($N = 500$) and the numbers remaining at termination of the experiment at 28 dph after discounting for those sampled during the experiment (approximately 40 to 50 per week). The resulting tally of live larvae and sampled (but censored) larvae was converted to proportions surviving.

2.4.2 Size and shape of larvae

Image-based size and shape of larvae reflect growth, condition, and developmental status. The weekly collection of larvae from each replicate population on each sampling day was analyzed for morphological features that varied with

age and development of the larvae. The measurements included lengths (total, standard, precaudal, flexion, mandible, and yolk), depths (total body, muscle mass at vent, and yolk), oil globule diameter (average of two orthogonal diameters), and a developmental indicator (flexion angle). Eight variables were used for samples taken of 0 and 28 dph larvae (Fig. 1). Three of eight variables (yolk length, yolk depth, and oil globule diameter) were unique to 0 dph larvae and two of eight (flexion length, flexion angle) were unique to age 28 dph larvae. Five variables were shared among larvae of all ages: total length, standard length, precaudal body length, total body depth at vent (including finfold or fin integument), and muscle mass depth at vent. Mandible length was included in the set of measures of larvae of all ages except 0 dph larvae. Morphometric data were extracted from images using UTHSCSA Image Tool software (http://compdent.uthscsa.edu/dig/itdesc.html).

2.4.3 Histopathology

Common ELS toxicities in fishes include cranio-facial and skeletal deformities, reduced growth, and cardiac disruption (Barron et al., 2003). Evidence of these and other possible CO_2 effects was examined by clearing and staining and by histopathological analysis of tissues and organs. The effects on cranio-facial and skeletal elements were assessed by alcian-blue staining of cartilage. The staining protocol entailed larvae being transferred to 70 % ethanol for 24 h to dehydrate the tissue, rinsed three times with 0.1 % Tween 20, and placed in a 2 µm syringe with filtered 0.1 % alcian-blue, 8GX (Sigma-Aldrich) stain overnight. Larvae were next placed into acidified ethanol to clear excess staining and then transferred to increasing concentrations of glycerol (20, 50, 80, and 100 %) with each step held for 15 min duration. The larvae were retained in 100 % glycerol to preserve the stain for photography and measurements. Each larva was inspected and measured with a SZ PT Olympus stereo microscope. Cranio-facial measurements taken were mandible length (tip of the dentary bone to the quadrate bone and to the end of the preopercular bone), lower jaw (tip of the dentary bone to the posterior end of the quadrate), upper jaw (maxilla), and snout length (tip of the premaxilary bone to the anterior cranium), where the nomenclature follows Wagemans and Vanderwalle (2001). Total length and cranio-facial structures (mandible, lower jaw, maxilla, and snout length) were measured at 1.1 × and 2.4 × magnification, respectively. Abnormal cartilaginous structures or staining characteristics were noted. The slide preparation resulted in five to six fishes from each replicate CO_2 group at each weekly sample as the basis for the cranio-facial analysis.

The effects of CO_2 level on soft tissue and organs were assessed by standard histopathological methods. The larvae were removed from their CO_2-exposure containers and immediately fixed in 10 % buffered formalin. Following fixation the larvae were processed through a standard dehydration and paraffin-embedding protocol. Biopsy cassettes were used for processing because of the small size of the larvae with three to four larvae placed in each cassette. The paraffin-blocked tissue was serially sectioned at approximately 5 µm increments, and every other slide was stained using hematoxylin and eosin (H&E). Organs and tissues analyzed included cranio-facial structures, eye, heart, liver, gall bladder, gastro-intestinal (GI) tract, epidermis, kidney, spinal cord, and muscles. The histopathology sections were examined for differences in tissue and cellular morphology by light microscopy. The evaluation of slides was performed using a double-blind protocol in which the evaluators had no

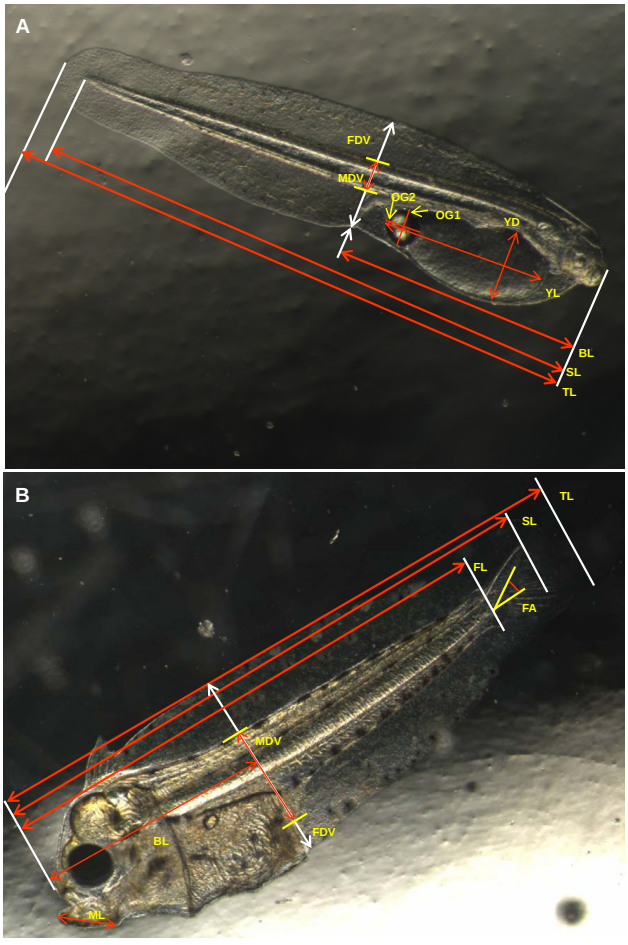

Fig. 1. Morphological variables used to describe size, shape, and development of summer flounder larvae in CO_2 effects experiment. Data were extracted from images of anesthetized live larvae in lateral perspective. The variables measured varied with larval age. **(A)** Yolk-sac larva at 0 dph. **(B)** Advanced larva (28 dph) undergoing notochord flexion. Abbreviations: total length (TL), standard (or notochord) length (SL), flexion length (FL), precaudal body length (BL), total body depth at vent (including finfold or fin integument) (FDV), muscle mass depth at vent (MDV), yolk length (YL), yolk depth (YD), oil globule diameters (OG1, 2), mandible length (ML), flexion angle (FA).

knowledge of the CO_2-treatment group (only the collection date and the assigned accession numbers were used for bookkeeping). A second trained pathologist, also blind to treatment group identities of samples, was used to confirm tissue and organ abnormalities. The CO_2-treatment key was not revealed to evaluators until after the slides had been scored for histological effects. The histopathological preparation analyses resulted in three to four fishes from each replicate CO_2 group at each weekly sample as the basis for summation of histological analyses of CO_2 effects on tissues and organs.

2.5 Statistical analyses

All statistical testing addressed the null hypothesis of no effect of CO_2 levels on the response variables. The statistical tests applied to these data were predicated on the assumption of independence of observations. In order to meet this assumption, either only one datum was analyzed per group from a shared container (e.g., analysis of the treatment × replicate means, medians, or summary statistics such as survival) or the interdependence of response variables was explicitly accounted for by applying multivariate statistical methods (e.g., multivariate analysis of variance, ANOVA).

For the embryo subexperiment, the three containers per treatment level available were used for the separate sibgroups and treated these sibgroups as replicates in the test of the null hypotheses. Space limitations precluded also replicating within females, but from prior experience with this species it was expected that the interfemale differences in egg batch quality and embryo survival would be large. Hence, pooling embryos across sibgroups and using the pool as the source of replicates would be ill advised as it would inflate the error variance and prevent any interpretation of that variance. The trade-off is that the intentional confounding of sibgroups and replicates results in the error variance for the test statistic including any among-sibgroup variance. Given the expected interfemale differences in egg batch quality, this trade-off was deemed acceptable. Moreover, such confounding parallels the action of replicating over time or blocking over space when the study logistics force a design in which replicates are initiated at different times or locations. There as here, confounding of replicates with an extraneous factor (time, space, or another system attribute) results in a conservative test of the effect of the treatment (e.g., CO_2 level) because the response variance associated with the extraneous factor is captured by the error term of the test statistic, making it less likely that the null hypothesis is rejected. Conversely, if the null hypothesis is rejected, it can be concluded that the treatment has a robust effect on the responses analyzed. Replicates for the larval subexperiment were drawn from a pooled set of hatchings. The critical value for statistical significance was set at $p = 0.05$. Analyses were conducted with SYSTAT 11 (SYSTAT Software, 2004) software package.

2.5.1 Survival data

Before analysis, proportions of embryos surviving to hatching were converted to the relative survival proportions for each replicate (= sibgroup) in order to standardize any interfemale differences in survival of their embryos. These embryo relative survival proportions, as well as the proportion of larvae surviving to the end of the experiment, were transformed via arcsine square root to normalize variances and then analyzed as one-way ANOVAs with CO_2 level as the treatment.

2.5.2 Size and shape data

All analyses of size and shape (morphometric) data were conducted on mean values per replicate within CO_2 levels. The statistical analysis of morphometric data was a three-step process. First, due to the lack of independence and expected covariance of variables describing size, shape, and developmental status of the same individual, these data were reduced by principal component (PC) analysis. Second, the resulting PC scores of individual fishes were reduced to replicate means. Third, the replicate mean PC scores were analyzed as an ANOVA or MANOVA depending on whether one or more PC axes were significant, respectively. Follow-up univariate tests for specific CO_2-treatment level contrasts, graphical display, and interpretation in the context of the original variables were conducted using ANOVAs.

2.5.3 Histopathology data

The data on H&E-stained responses of cartilage and skeletal elements (i.e., total length and cranio-facial features) were handled similar to the image-based size and shape data (above). Those data were subjected to a PC analysis to reduce dimensionality of correlated variables and then submitted to one-way (M)ANOVA to test for no effect of CO_2. The histopathological responses of tissue and organ responses are reported in a qualitative manor for each CO_2-level group and age.

3 Results

3.1 CO_2 effects on survival

The proportion of fertilized eggs surviving to hatching decreased with increasing CO_2 and water acidity (Fig. 2, Table S2). The relative survival to hatching was reduced by approximately half (0.48) as CO_2 increased from low (ambient)-CO_2 conditions to intermediate-CO_2 levels (2.5 times ambient), and reduced again by more than half (0.16 of initial number of embryos) at the high-CO_2 level (6 times ambient). The decrease in the proportion surviving to hatching due to CO_2 was marginally significant on the absolute scale ($F_{2,6} = 4.4$, $p = 0.07$, $R^2 = 0.60$) despite differences

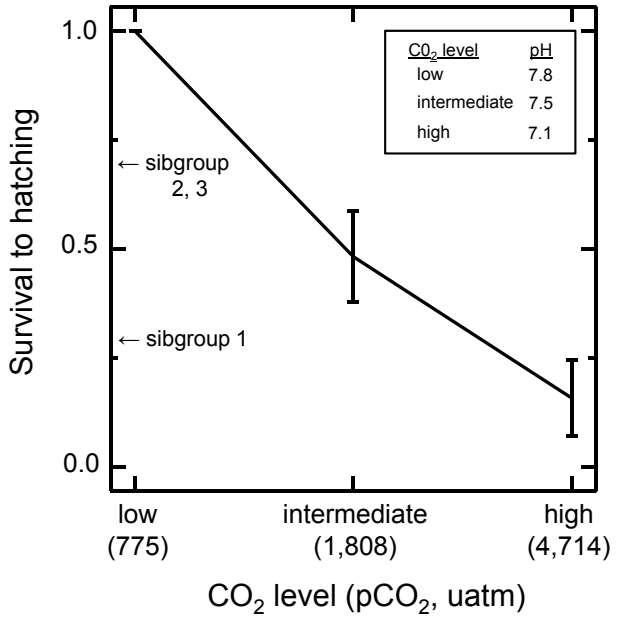

Fig. 2. Proportion of fertilized eggs of summer flounder surviving to hatching at different levels of CO_2 in rearing water (inset: associated pH values). Embryos were obtained from three females each crossed with milt pooled from three to five males. Mean (±SE) relative survival is plotted where survival data for each female sibgroup were first scaled to sibgroup survival at ambient (low) CO_2 (i.e., relative survival at low $CO_2 = 1.0$ for all sibgroups). Absolute survival at ambient conditions for each sibgroup is denoted by arrows.

among sibgroups from different females in survival at low CO_2 (range of survival to hatch 0.29 to 0.70, Fig. 2, arrows). Once interfemale differences were accounted for by computing relative survival, the decrease due to CO_2 level was highly significant ($F_{2,6} = 31.0$, $p = 0.001$, $R^2 = 0.91$). This pattern of reduction in survival was consistent across all three replicates (sibgroups), which justifies retaining sibgroup integrity during the embryonic subexperiment. Survival to hatching also decreased significantly between the low- and intermediate-CO_2 levels (two-group contrast, $F_{1,4} = 70.5$, $p = 0.001$, $R^2 = 0.95$).

Survival of larvae to the termination of the experiment (28 dph) ranged from 0.64 to 0.91. There was no effect of CO_2 levels on larval survival (Table S2).

3.2 Size, shape, and development of larvae

3.2.1 Variance structure of larval morphological variables

The expectation that the morphological variables within individuals at each sample age were interdependent and correlated was confirmed by PC analysis. The analysis was run on each weekly sample and provided one or more significant independent linear combinations (PC) from the original variables. The PC scores captured well the variance structure of the original data and offered insights into how larval size, shape, and developmental status changed as a function of age and CO_2 level. For 0 and 14 dph larvae, only one PC ($PC1_{0\,d}$ and $PC1_{14\,d}$) was significant, and it accounted for 69 and 85 % of the original among-individual variance, respectively. For 28 dph larvae, two PCs ($PC1_{28\,d}$, $PC2_{28\,d}$) were significant (Fig. 3), and they accounted for 78 and 17 % of the variance, respectively (sum of 94 %).

For larvae at each of the three sample ages shown here (0, 14, and 28 dph), larval size appears to be the best characterization of PC1 (PC loadings onto PC1 and PC2 for 28 dph larvae are shown in Fig. 3; values of loadings onto PC1 for 0, 14, and 28 dph are in Fig. 4, inset, and Table S2). Larval lengths (total, standard, and body lengths for all ages, but also including flexion lengths for older larvae) were consistently major contributors to PC1. Measures of body depth of the larvae, especially at older ages, and mandible length also contributed positively to PC1. The consistently large loadings of measures of fish lengths and body depths (all with same positive sign) support a general depiction of isometric growth independent of absolute size; i.e., larger (smaller) larvae are proportionately larger (smaller) in all of these measures taken at all ages. For 0 dph larvae, an inverse relationship existed between larval size and energy reserves at hatching. The measures of energy available to the larvae (yolk length and depth, and oil globule diameter) contributed negatively to PC1 (Fig. 4a and Table S2). For advanced larvae (28 dph) the developmental events in late larval ontogeny contributed most to PC2. Specifically, the extent of flexion – quantified by flexion angle (Fig. 1) – along with a secondary contribution by the depth of body musculature load most heavily on PC2 (Fig. 3). These developmental events near the terminus of the larval period – increased degree of flexion and a deepening of the body – reflect imminent metamorphosis.

3.2.2 CO_2 effects on larval size and development

Elevated levels of CO_2 affected larval size and development. The pattern of effects changed with larval age, was evident in the PC-reduced scores, and is reflected repeatedly in the original morphological variables (Figs. 4 and 5, Table S2). At hatching, larvae from embryos maintained at low-CO_2 conditions were significantly smaller in size (length and depth) but had more yolk and larger oil globules than larvae from embryos maintained in higher-CO_2 environments, including those from the intermediate-CO_2 level. The CO_2 effect is reflected in the PC1 scores for 0 dph larvae (Fig. 4a). This pattern is also evident in any measure of size (length or body depth) of 0 dph larvae and in the inverse response in any measure of their energy reserves (e.g., total length and yolk length of 0 dph larvae are inversely related, Fig. 5a). Again, significant differences were evident in size and energy reserves between larvae from the low- and intermediate-CO_2 environments.

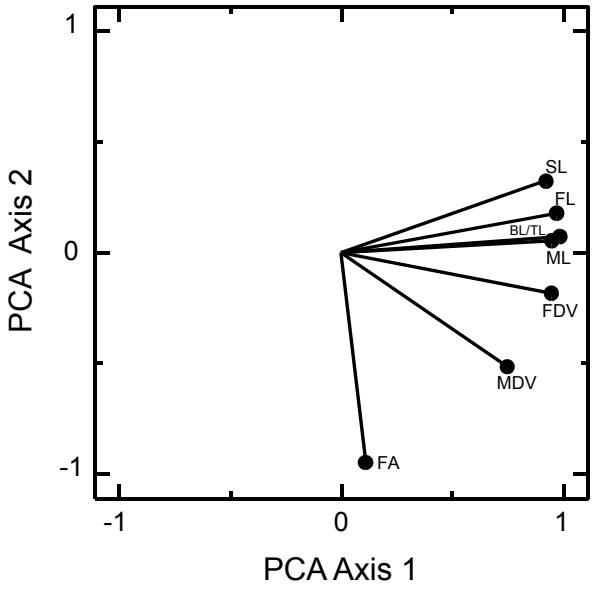

Fig. 3. Contribution (loadings) of morphological variables measured on late-stage summer flounder larvae (28 dph) to significant principal component (PC) axes. PC axis 1 accounted for 77 % of overall variance and is dominated by measures of fish size (primarily length but also body depth measurements). PC axis 2 accounted for an additional 17 % of the remaining variance and largely reflects developmental stage (degree of flexion) with additional contributions by depth of body muscle mass. See Fig. 1 for abbreviations and Table S2 for summary statistics.

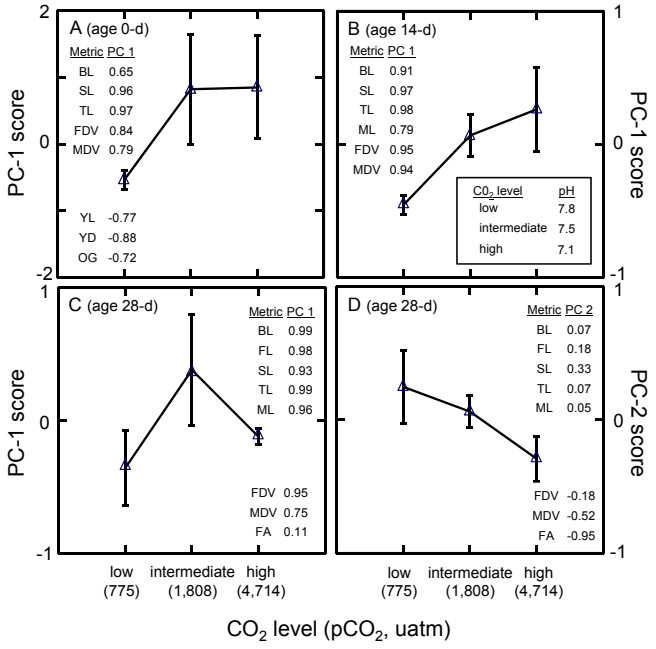

Fig. 4. Responses in size, shape, and development of summer flounder larvae by age at different levels of CO_2 in rearing water (inset: associated pH values). Responses are summarized by principal component (PC) scores. Only significant PC axes are shown. **(A)** 0 dph, PC 1. **(B)** 14 dph, PC 1. **(C)** 28 dph, PC 1. **(D)** 28 dph, PC 2. The PC loadings on each original measurement (metric) are shown at each age within panels. Data plotted are means ±SE. See Fig. 1 for abbreviations and Table S2 for summary statistics.

This pattern of smaller larval sizes (lengths, depths, mandible size) at low-CO_2 conditions compared to larvae from intermediate- and high-CO_2 environments continued through the mid-larval period samples at 7 and 14 dph (data from 14 dph larvae shown in Fig. 4b). By 21 dph (not shown in Figures) the size ranking of larvae from CO_2 environments began to shift, with larval sizes comparable among all CO_2 environments but with a trend towards smaller sizes at the high-CO_2 environment. By 28 dph (the last trimester of the larval period at the 19.5 °C rearing temperature), the larvae at the intermediate-CO_2 environment were the largest sizes while larvae from the low and high-CO_2 environments were smaller and comparable in size (Figs. 4c and 5b). Despite a rough size equivalency between larvae from low and high-CO_2 environments, these larvae were at significantly different developmental stages (Fig. 4d). Larvae from the low-CO_2 environment were the least advanced of all groups in terms of notochord flexion, those from the intermediate-CO_2 environment were intermediate in their development, and larvae from the highest-CO_2 environment were the most developmentally advanced at the termination of the experiment. Indicators that metamorphosis was imminent in the larvae from the high-CO_2 environment were the greater degree of notochord flexion (Fig. 5c), deepening bodies, and prevalence of eye asymmetry.

3.3 CO_2 effects on histopathology features

The alcian-blue staining allowed quantification of skeletal elements, including the lengths of the total body, mandible, lower jaw, maxilla, and snout of larvae ≥ 7 dph (younger larvae could not be reliably prepared for histological examination). Total length and all four cranio-facial measurements were interrelated, contributing in similar magnitude to the single significant PC (Fig. 6, Table S3). The ontogenetic trajectory of each of the original measures and PC1 scores mirrored that of the changes in the size variables based on external morphometric measurements presented above. Specifically, larvae from the high-CO_2 level tended to have larger cranio-facial features up through the mid-larval period. By age 21 dph and continuing through 28 dph, the larvae from the low-CO_2 level had the largest cranio-facial features. In addition, larvae from the high-CO_2 environment showed occasional deformations such as delayed formation of the mandible.

The histopathology evaluations revealed that all larvae examined (age ≥ 7 dph) had food in their GI tracts. No significant lesions were observed in the GI tract, pancreas, gill, eye, kidney, and heart of larvae from any CO_2 environment at any age. Livers of larvae from the low and intermediate-CO_2

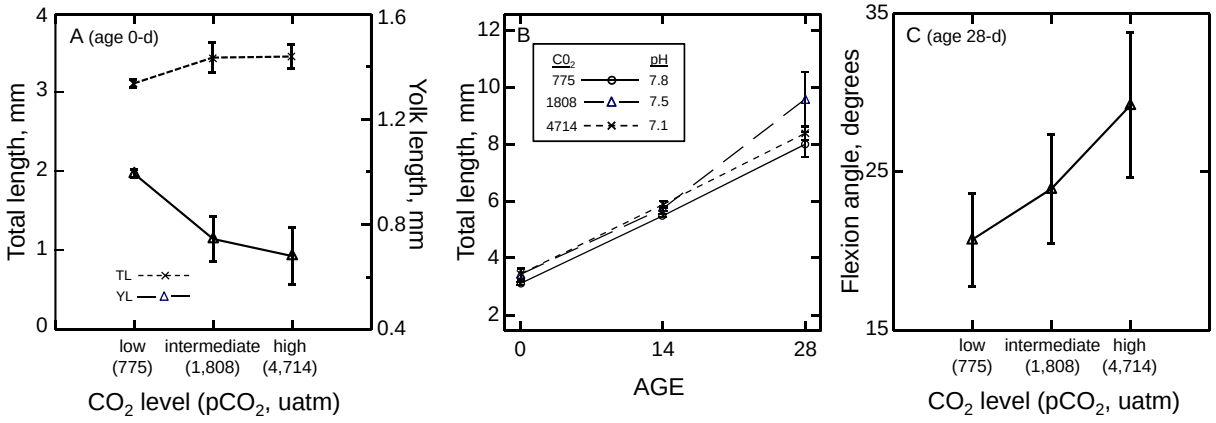

Fig. 5. Responses of summer flounder larvae to different levels of CO_2 in rearing water (inset: CO_2 levels (µatm) and associated pH values). **(A)** Total length (TL) and yolk length (YL) of larvae at hatching (0 dph). **(B)** Total length versus age of larvae. **(C)** Notochord angle of advanced larvae (28 dph) as an indicator of larval developmental stage. Flexion angle (see Fig. 1b) increases from zero as metamorphosis is approached. Data plotted are means ±SE.

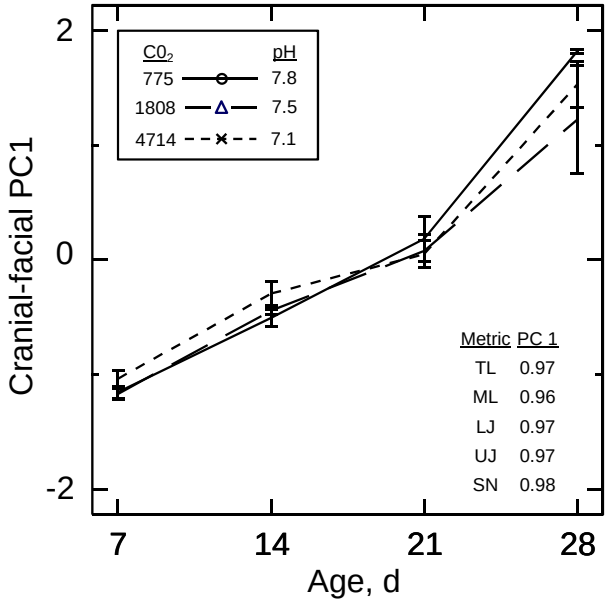

Fig. 6. Responses in cranio-facial features of summer flounder larvae by age at different levels of CO_2 in rearing water (inset: CO_2 levels (µatm) and associated pH values). Responses are as summarized by the first principal component (PC) scores. The PC loadings on each original cranio-facial measurement (metric) are shown within panel. Plotted data are means ±SE. See Sect. 2.4.3 for description of measurements and abbreviations, and Table S3 for summary statistics.

environments were similar in appearance; however larvae from the intermediate-CO_2 environment tended to express minor liver sinusoid dilation. Larvae from the high-CO_2 environment expressed additional abnormalities including minor focal hyperplasia on the epithelium and separation of the trunk muscle bundles (Fig. 7). In addition, extensive dilation of the liver sinusoids and central veins was evident, which resulted in the liver appearing condensed and being organized into rows or patches.

4 Discussion

4.1 Experimental protocol and CO_2 levels

Our experiments revealed the embryos and larvae of summer flounder to be sensitive to elevated-CO_2 levels and that the effects on survival, size, and development occurred even at the intermediate-CO_2 levels used here. Two points on our experimental protocol warrant emphasis and help to place these results in context.

First, this study manipulated the levels of CO_2 in our experiment test containers in order to simulate future ocean conditions (intermediate-CO_2 level used here) and to test for response to an extreme level of CO_2 (the high-CO_2 level). We chose to infuse the seawater in the experimental system with CO_2 to lower acidities indirectly rather than lowering acidities by addition of acids. Our protocol more closely simulates the process of OA in nature and conforms to recommended best practices in OA studies (Riebesell et al., 2010). By using this protocol of infusion of CO_2, however, we cannot distinguish between the effects of a more acidic environment caused by elevated CO_2 and the direct, non-pH effects of CO_2 on the physiology of young summer flounder. As an example of the latter, a consequence of CO_2 binding with, and thus changing the conformation of, hemoglobin is that hemoglobin's affinity to oxygen is reduced. This alteration, known as the Bohr effect, may have significant consequences of its own. Unless CO_2 level and acidity are elevated independently – which was not done here – these separate consequences cannot be evaluated.

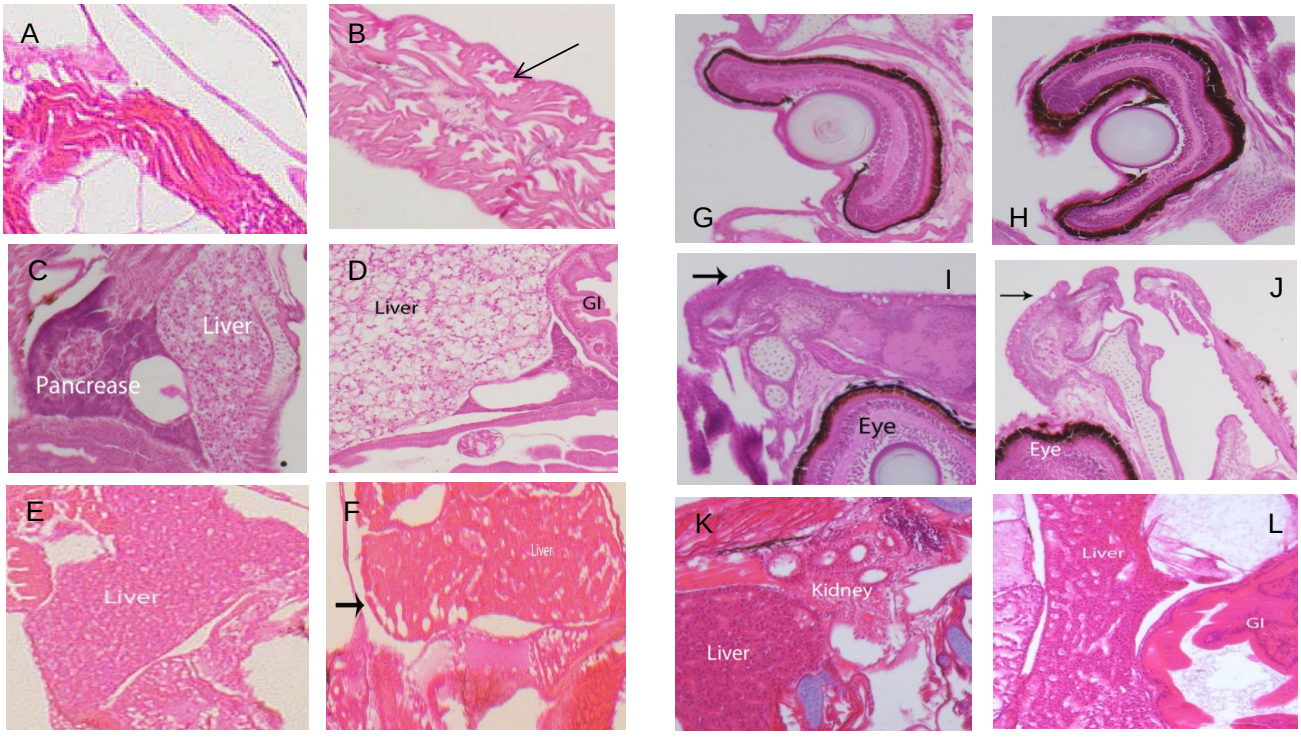

Fig. 7. Photomicrographs of H&E-stained tissue sections taken from 21 dph **(A–F)** and 28 dph **(G–L)** summer flounder larvae. Photomicrographs on left **(A, C, E, G, I, K)** are from ambient (low)-CO_2 level; those on right **(B, D, F, H, J, L)** are from the high-CO_2 level. **(A, B)** Skeletal muscle. **(C, D)** Liver (lipid or glycogen vacuolation) and pancreas. **(E, F)** Liver (eosinophilic hepatocytes and chord-like structure). **(G, H)** Eye showing cornea, lens, iris, and retina. **(I, J)** Epidermis. **(K, L)** Kidney (renal tubules) and liver. Notes: **(B)** arrow shows disruption of skeletal muscle fibers within the bundles. **(C–F)** Livers of some 21 dph larvae had a vacuolated appearance **(C, D)** and others had a more distinct chord-like architecture **(E, F)**, but in both types the liver architecture in the high-CO_2 group was disrupted and more apparent as dilated sinusoids (**F**, arrow) and focal hepatocyte necrosis. **(G, H)** No significant differences among groups were observed in the eye. **(I, J)** Epidermal hyperplasia was evident with a marked increase in goblet cells (arrows). **(K, L)** Livers from the 28 dph high-CO_2 group **(L)** showed dilated sinusoids and focal necrosis when compared to the low-CO_2 group **(K)**.

Second, the CO_2 levels that we used met our criteria for a step-one experiment as described above (see General approach). The intent of such an experiment is to screen species and response variables for their sensitivity to future and extreme CO_2 levels. Although high-resolution CO_2 data for inshore habitats are unfortunately quite sparse for most regions worldwide – and the Mid-Atlantic Bight is no exception – there is recent evidence and there are suggestions from historic surveys (e.g., MARMAP 1978–1987) that temperate and higher-latitude inshore regions, including the Mid-Atlantic Bight, are likely to have higher and more variable CO_2 levels than those predicted for open-ocean environments (Duarte et al., 2013; Wang et al., 2013). The Mid-Atlantic Bight as a whole is a net sink for atmospheric CO_2, with the annual cycle of heating and cooling combined with high winds during the period of undersaturation (winter) accounting for a significant portion of the uptake of CO_2 (DeGrandpre et al., 2002). The marine shelf habitat of the Mid-Atlantic Bight has substantial spatial (nearshore–offshore) and seasonal variation in CO_2 levels, with nearshore habitats in summer-autumn exhibiting mean bottom-water pCO_2 values as high as 722 µatm (D. Wieczorek, unpublished data, 2008–2010). Some summer flounder spawn at this time and their ELS occupy these habitats. Hence, the low-CO_2 level used in experiments here are tailored to the study species. As argued by McElhany and Busch (2013), such local conditions provide a more realistic benchmark in OA experiments than open-ocean averages.

Although the shelf waters of the Mid-Atlantic Bight are stable with respect to high-frequency CO_2 variations relative to those observed in estuarine habitats, summer flounder ELS could be exposed to CO_2 levels as high as our low-CO_2 experimental treatment of 775 µatm. Our intermediate-CO_2 level (1860 µatm) represents a 2.4-fold increase above the inshore autumn benchmark of 775 µatm. This intermediate value also is just under an 80 % increase above the IPCC prediction for open oceans by 2100, and close to the IPCC projections for 2300. The high-CO_2 level used here (4714 µatm) was set high in order to establish whether an effect could be elicited in our various response variables at

extreme conditions. Importantly, many of the effects reported here were exhibited even at the intermediate-CO_2 level.

4.2 CO_2 effects on embryo survival

A strong effect of CO_2 was evident in the survival to hatching of summer flounder embryos. This pattern of reduction in relative survival by half with a 2.5 times increase in CO_2 levels (0.35 pH unit reduction) in the environment, and a further reduction by more than half again with another 2.5 times increase above the intermediate-CO_2 levels (a further 0.4 pH unit reduction), was consistent among three sets of embryos from different parents. A reduction in the number of viable hatching larvae by even 50 % as found for the intermediate-CO_2 environment (1808 µatm, pH 7.5) is unlikely to be sustainable as this reduction would be chronic unless summer flounder can responds via acclimation, nongenetic transgenerational effects, or natural selection to the CO_2 levels predicted for late this century and through the next one.

A positive covariance between maternal CO_2 environment and the performance of offspring has been reported for tropical reef fishes (Donelson et al., 2012; Miller et al., 2012). It is noteworthy that we saw significant responses of offspring to elevated-CO_2 environment even though their parents were maintained in local ambient seawater with a pH of 7.46 to 7.63 prior to spawning. All of the prespawned adult fishes were maintained at local ambient conditions, so we could not evaluate the effects on offspring of adult conditioning to varying CO_2 environments. Although we did not attempt to estimate heritability of resistance to elevated-CO_2 environments, and thus the potential for selective response was not assessed, the protocol here of retaining family integrity for the embryo subexperiment does allow a glimpse at the likelihood of the potential for a selective response. The finding that these separate sibgroups responded nearly identically in their proportional reduction of embryo survival with increasing CO_2 environments is consistent with a limited potential to respond via natural selection to future elevated-CO_2 environments. Further study is needed to rigorously evaluate the role of parents in providing advantages to their offspring via maternal conditioning to elevated-CO_2 environments or through heritable variation in tolerance to these environments.

The degree of reduction in embryo survival at elevated-CO_2 environments found here has not been reported by most other authors whose combined work is on a diverse set of marine fishes. Munday et al. (2009b) found the survival to hatch of orange clownfish (*Amphiprion percula*) from the Great Barrier Reef, Australia, to be nonresponsive to pCO_2 levels up to 1030 ppm. Franke and Clemmesen (2012) found no significant effect of elevated pCO_2 (460 to 4635 ppm) on survival to hatch of Atlantic herring from parents collected in the western Baltic Sea. Frommel et al. (2013) found that survival of embryos of Atlantic cod from parents collected in the Bornholm Basin of the western Baltic Sea was not altered at pCO_2 levels up to 4000 ppm. Hurst et al. (2013) report no effect on embryo survival of walleye pollock, common in the temperate eastern North Pacific, at pCO_2 levels up to 1933 ppm. In contrast, Baumann et al. (2012) reported a 74 % reduction in survival of embryos and young larvae of inland silverside, *Menidia beryllina*, native to estuaries of the US Atlantic coast, when maintained at higher pCO_2 levels (1100 ppm) compared to those held at lower pCO_2 levels (410 ppm). All of these studies varied in the number of parents used, the time lapse between egg fertilization and the initiation of the CO_2 treatments, and in how and when survival was scored. For example, the CO_2 exposures of inland silverside by Baumann et al. (2012) began at approximately 24 h post-fertilization, and survival was scored at approximately 1 week post-hatching.

The different protocols used among previous studies may preclude a fair cross-study comparison; however, the overall lack of effect of elevated-CO_2 environments on embryo survival (with the conditional exception of inland silverside by Baumann et al. (2012)) is in contrast to the findings here. The habitats occupied by a species, particularly its ELS, may play a role in their sensitivities to elevated-CO_2 environments. Why embryos of two species whose ELS are found in estuarine (inland silverside) or inner shelf (summer flounder) habitats, both with relatively high ambient-CO_2 levels, exhibit sensitivity to experimentally elevated-CO_2 levels is counter to expectations and requires further attention.

4.3 CO_2 effects on larval size, condition, and development

A consistent trend in the effects of elevated-CO_2 environments on larval summer flounder ontogenetic trajectories was evident – one that is ecologically important and could negatively affect recruitment. Larvae from elevated-CO_2 environments were larger at hatching but had less energy reserves (smaller yolk and oil globule) than larvae from low-CO_2 conditions. Such a trade-off between size and energy reserves of larvae at hatching from contrasting environments (e.g., two or more thermal regimes or maternal sources) has been exhibited in other marine fishes (Chambers et al., 1989). Here the trade-off means that larvae from environments with higher CO_2 would be larger but likely have less time to successfully initiate feeding.

All else equal, large size is thought to confer an advantage to larvae via enhanced prey capture and/or predator avoidance (Miller et al., 1988). Greater larval lengths and growth rates in CO_2-enriched environments have been observed in Atlantic cod (Frommel et al., 2012) and walleye pollock (Hurst et al., 2013), but the opposite pattern was reported for inland silverside (Baumann et al., 2012). Importantly, and countering the presumed benefits of large size, Frommel et al. (2012) found that larval Atlantic cod from high-CO_2 environments were longer but in poorer condition and more likely to display tissue and organ damage than larvae from low-CO_2 environments – a relationship also found here

with summer flounder. Using another measure of condition, Franke and Clemmesen (2011) report that larvae of Atlantic herring from high-CO_2 environments were of lower condition (i.e., lower RNA-to-DNA ratios) than their counterparts from lower-CO_2 environments.

Summer flounder larvae from low-CO_2 environments in experiments reported here were smaller than those from high-CO_2 environments until at least the mid-larval period. An inverse relationship between CO_2 levels in the environment and the duration of the larval period was also evident. Specifically, the growth trajectories and developmental stages at the termination of this experiment (28 dph) show evidence that larvae at low-CO_2 levels can be expected to be larger and older at the completion of metamorphosis than those from the high-CO_2 levels and quite likely larger than those from the intermediate-CO_2 level.

Few other OA studies have considered a multivariate characterization of larval size, shape, and development with which to compare results from this study, and none has considered flatfish – a taxon whose ontogeny conforms exceedingly well to scoring of developmental progression during the larval period. An intriguing study by Munday et al. (2011) provides a highly multivariate characterization of possible CO_2 responses in the early life history of spiny damselfish (*Acanthochromis polyacanthus*). Fishes were experimentally exposed to a range of pCO_2 levels (450 to 850 ppm) beginning on hatch day. They evaluated 29 skeletal elements from cleared-and-stained specimens. Three of 29 elements differed significantly (one-way ANOVA) among CO_2 levels, but none was monotonically related to CO_2 level and no skeletal element varied significantly among CO_2 levels after the authors applied a Bonferroni correction to the ANOVA test critical value in order to accommodate multiple tests on their data set. Munday et al. (2011) evaluated other responses (survival, fish length and mass, and otolith morphometry) and found none to vary with CO_2 level. This lack of sensitivity to CO_2 challenges may be related to the life history of the spiny damselfish, which lacks a true larval period and hatches as a young juvenile.

An altered ontogenetic trajectory in response to an elevated-CO_2 environment as found in this study may be of considerable ecological importance in summer flounder. Because this species primarily spawns in autumn (September to December), its larvae experience rapidly declining water temperatures. Depending on the timing of spawning, the autumn sea temperatures, and the intrinsic ontogenetic rate, larvae from a single summer flounder annual cohort may metamorphose, ingress, and settle into estuaries before winter, after winter, or both (R. C. Chambers, unpublished data). Importantly, larvae are incapable of metamorphosing at the cool winter water temperatures of this region (December through March), so acceleration of developmental timing as a consequence of elevated-CO_2 levels would amplify any consequences – or benefits – of ingressing in the autumn versus spring.

4.4 CO_2 effects on tissues and organs

The number and severity of malformations in tissues and organs of summer flounder larvae increased with levels of CO_2 in the environment. The majority of these differences were seen in older larvae, especially in the 21 and 28 dph samples which showed significant differences between the highest and lowest-CO_2 environments. The fewer observed abnormalities in younger larvae (e.g., 7 dph) may be due to their small size (3.4 to 3.7 mm TL) and difficulties in preparing and accurately measuring these larvae. A trend towards reduction in frequency of abnormalities with age may reflect mortalities of severely impaired larvae before the sample date. Selective and age-dependent mortality in the larval period, possibly as an increasing fraction of the surviving older larvae exhibit homeostatic capabilities that accommodate high-CO_2 levels as found in Atlantic cod (Frommel et al., 2012), may account for some of the reduction in frequency of developmental anomalies seen here for larvae at advanced ages.

Although the extent of cranio-facial abnormalities lessened in older larvae, the trend towards reduced sizes of cranio-facial features in high-CO_2 environments was evident in larvae at the most advanced sample date (28 dph). Indeed, the lengths of all cranio-facial measures in advanced-age larvae tended to be smaller for fishes from the high- versus low-CO_2 environments. Part of these differences among larvae from different CO_2 groups may reflect the different (accelerated) developmental stage of the high-CO_2 group and the remodeling of the fish cranium that occurs at metamorphosis. The extent, location, and proximate cause of high-CO_2-induced abnormalities of the cranio-facial areas of marine fish species is largely unknown and has not been previously evaluated in summer flounder. Like all flatfish, summer flounder larvae undergo metamorphosis during which the head is remodeled and, in this species, as the right eye of the larva migrates to the left side of the face (Schreiber and Specker, 2000). While this morphology has an evolutionary benefit (Schreiber, 2006), the massive change of cranio-facial structures likely renders the remodeling process as highly sensitive to environmental stressors. The metamorphosis of flatfish is influenced proximally by thyroid hormone (TH), which also controls the tolerance of the fishes to changes in salinity (Schreiber, 2006). The influence of elevated-CO_2 levels and increased acidity of seawater on the production of TH in flatfish and the responsiveness of the target tissues to TH warrant further investigation.

5 Conclusions and prospective

A dramatic reduction in survival of summer flounder embryos in elevated-CO_2 environments was found. The CO_2 effects on larval size, condition, and developmental rates were more subtle and were revealed only through a holistic

multivariate approach. Negative effects of a high-CO_2 environment were also evident in larval tissues and cranio-facial features and, like the CO_2 effects expressed in the external phenotypic measures, changed with age and development. Importantly, the observed CO_2-induced variations – even those at the intermediate, next-century CO_2 values used here – have predictable negative consequences on recruitment of this ecologically and economically important species.

A growing understanding of OA effects on marine fishes is taking shape in the research community. This understanding acknowledges the challenges in drawing generalizations when confronted with OA studies on diverse taxa that use variable experimental protocols and employ different types and ways of measuring biological responses. A research strategy that may produce rapid progress on OA research was offered above (General approach), and the first step of that strategy was used in this study.

Three additional criteria for broadening an OA research strategy warrant consideration.

1. Select experimental subjects with life histories, ecologies, and habitat affinities that are representative of broader species groups. Given the infancy of OA research on marine fishes, the OA community is limited by the small number and biases of taxa studied to date. The species chosen for past studies have likely been of convenience and familiarity rather than ones that could provide stronger inferences.

2. Identify best practices, refine methods where needed, and document all protocols. Due to the international and multijurisdictional interest in OA, the research community has recurring opportunities for workshops, symposia, and best-practices guidelines (e.g., Riebesell et al., 2010).

3. Provide OA project metadata to the OA research communities. Interstudy comparisons and retrospective studies are only as good as the data and metadata available for such analyses. To that end, multiple agencies – including NOAA's Ocean Acidification Program – are developing metadata standards. Input to these standards as well as critiques and usages of them are needed in order to maximize their utility for the largest fraction of the OA research community, resource managers, and the concerned public.

Supplementary material related to this article is available online at http://www.biogeosciences.net/11/1613/2014/bg-11-1613-2014-supplement.pdf.

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