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Ocean acidification effects in the early life-stages of summer flounder, *Paralichthys dentatus*

R. C. Chambers[1], A. C. Candelmo[1], E. A. Habeck[1], M. E. Poach[1], D. Wieczorek[1], K. R. Cooper[2], C. E. Greenfield[2], and B. A. Phelan[1]

[1]Howard Marine Sciences Laboratory, NOAA Fisheries Service, 74 Magruder Rd., Highlands, NJ 07732-4054, USA
[2]Department of Biochemistry & Microbiology, Rutgers, The State University of New Jersey, 76 Lipman Dr. – Room 218, New Brunswick, New Jersey 08901-8525, USA

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Correspondence to: R. C. Chambers (chris.chambers@noaa.gov)

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Abstract

The limited available evidence about effects of high CO_2 and acidification of our oceans on fish suggests that effects will differ across fish species, be subtle, and interact with other stressors. An experimental framework was implemented that includes the use of (1) multiple marine fish species of relevance to the northeastern USA that differ in their ecologies including spawning season and habitat; (2) a wide yet realistic range of environmental conditions (i.e., concurrent manipulation of CO_2 levels and water temperatures), and (3) a diverse set of response variables related to fish sensitivity to elevated CO_2 levels, water temperatures, and their interactions. This report is on an array of early life-history responses of summer flounder (*Paralichthys dentatus*), an ecologically and economically important flatfish of this region, to a wide range of pH and CO_2 levels. Survival of summer flounder embryos was reduced by 50 % below local ambient conditions (7.8 pH, 775 ppm pCO_2) when maintained at the intermediate conditions (7.4 pH, 1860 ppm pCO_2), and by 75 % below local ambient when maintained at the most acidic conditions tested (7.1 pH, 4715 ppm pCO_2). This pattern of reduced survival of embryos at higher CO_2 levels was consistent among three females used as sources of embryos. Sizes and shapes of larvae were altered by elevated CO_2 levels with longer larvae in more acidic waters. This pattern of longer larvae was evident at hatching (although longer hatchlings had less energy reserves) to midway through the larval period. Larvae from the most acidic conditions initiated metamorphosis at earlier ages and smaller sizes than those from more moderate and ambient conditions. Tissue damage was evident in older larvae (age 14 to 28 d post-hatching) from both elevated CO_2 levels. Damage included liver sinusoid dilation, focal hyperplasia on the epithelium, separation of the trunk muscle bundles, and dilation of the liver sinusoids and central veins. Cranial-facial features were affected by CO_2 levels that changed with ages of larvae. Skeletal elements of larvae from ambient CO_2 environments were comparable or smaller than those from elevated CO_2 environments when younger (14 d and 21 d post-hatching) but larger at older ages (28 d). The de-

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gree of impairment in the early life-stages of summer flounder due to elevated CO_2 levels suggests that this species will be challenged by ocean acidification in the near future. Further experimental comparative studies on marine fish are warranted in order to identify the species, life-stages, ecologies, and responses that are most sensitive to increased levels of CO_2 and acidity in near-future ocean waters, and a strategy is proposed for achieving these goals.

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 double from current open ocean concentrations (~ 370 ppm pCO_2) to over 750 ppm by 2100 with a decrease in surface water pH of ~ 0.4 units (IPCC, 2007). The baseline ambient and predicted levels of CO_2 and acidity of seawater are substantially greater at regional scales especially for continental shelf, nearshore, estuarine, and high latitude habitats (Duarte et al., 2013). These regions are also where a large fraction of the commercially extractable living marine resources resides.

Research on OA effects in marine fishes is in its infancy with the large majority of published accounts on this topic having appeared in the last five years. This small but growing body of studies on the OA effects in marine fish species is impressive in the diversity of study species, their native systems, and the broad array of response variables evaluated. The taxa used in prior studies include tropical reef fishes (Munday et al., 2009), a temperate sciaenid (Checkley et al., 2009), an estuarine antherid (Baumann et al., 2012), a gadid from the NE Pacific shelf (Hurst et al., 2013), and a clupeid (Franke and Clemmeson, 2011) as well as another gadid from the low salinity Baltic Sea (Frommel et al., 2012, 2013). The diversity of responses evaluated for evidence of CO_2 effects include sperm activity (Frommel et al., 2010) and early life-stage survival

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(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), olfactory capabilities (Dixson et al., 2010), sidedness (Domenici et al., 2011), homing ability (Munday et al., 2009a), and predator avoidance (Ferrari et al., 2011).

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

Few studies have addressed OA effects in marine fish species 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 Northwest 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 was designed to begin filling that information gap with fish species that are of economic and ecological importance to the mid-Atlantic region of the USA and is part of a larger research effort funded by the US National Oceanic and Atmospheric Administration (NOAA). Our objective was to identify and quantify the effects of elevated levels of CO_2 on the early life-stages of summer flounder, *Paralichthys dentatus*, an ecologically and commercially important paralichthyd flatfish common to inshore waters of this region.

This study focuses on the early life-stages (ELS) of summer flounder which, for this study, encompass the embryonic stage (fertilization to hatching) and most of the feed-

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ing larval stage (from hatching until initiation of metamorphosis into the juvenile morphology). The ELS are thought to be more susceptible to toxic substances and conditions than adults and juveniles (Woltering, 1984), are likely to be the life stages least capable of regulating internal acid-base balances, and therefore are likely to be most 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 and this is when year-class strength is determined.

General approach

This report represents results from one of a series of conducted or planned experiments on OA effects in marine and estuarine inhabiting fish species of the mid-Atlantic states of the USA. Beyond their importance to this ecosystem, the species were chosen to represent a diverse phylogenetic and ecological set. Using a comparative experimental framework allows generalizations about the relative likelihood of OA effects with respect to phylogeny, life history, ecology, and habitat.

The experiment implemented here is the first in a three-step sequence. Step-one experiments are one-way factorial designs with CO_2 concentration as the manipulated treatment. The range of CO_2 concentrations used is broad, spanning from current concentrations in local (New Jersey, USA), inshore ambient seawater to CO_2 levels associated with an approximate doubling to a those at an approximate quadrupling of these levels. The ~twice-ambient CO_2 target reflects IPCC predictions from some model scenarios for later this century and into the next one, while the highest CO_2 level used was intended as an extreme condition for which a lack of ELS response would support a robust conclusion of the resiliency of the test species to CO_2 challenges. Step-two experiments are two-way factorial designs with CO_2 concentration crossed with a second treatment that may act as a co-stressor and potentially interact with CO_2 in its effects on the test subjects. The ranges of treatments are again strategically broad. Step-three experiments are also two-way factorial designs (CO_2 concentration and co-stressor) but with the range of treatment levels restricted to those slightly above and

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below ones that elicited the steepest gradient of change in step-two designs. Results from this experimental progression should provide identification and quantification of responses due to treatment(s) that can be inspected for the functional form and parameter values, and hence should provide predictive equations of the responses of the ELS of marine fishes to environmental change.

The evaluated ELS characteristics of the test species in these experiments are intended to cover a wide array of lethal and sublethal responses. By identifying and providing estimates of the absolute and relative sensitivities of these responses to elevated CO_2 levels, this protocol will also offer guidance regarding the technical merit of candidate high-CO_2 responses for 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 shelf waters throughout the year with many ingressing to bays during the warmer months (April through September). Summer flounder becomes piscivorous as young-of-the-year juveniles and is an important predator in this ecosystem thereafter.

Summer flounder spawns buoyant eggs in continental shelf water in the autumn with peak spawning occurring from September to December. Summer flounder eggs and larvae experience seasonally cooling pelagic environments (spawning at ~22 to 14 °C and larvae inhabiting 22 to 8 °C seawater depending on spawning time and latitude). Eggs hatch within 2 to 7 d and larvae settle between 5 and 15 weeks depending on spawning time and local conditions. The pelagic waters occupied by summer flounder ELS are stable relative to nearby estuarine systems with respect to high-frequency temporal changes in CO_2 levels.

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2.2 CO_2 experimental implementation

Controlled levels of CO_2 were supplied to the experimental subjects at our laboratory by a large-scale, flow-through experimental CO_2-delivery system designed for our multi-species, three-step OA experimental framework. The system consists of a pre-treatment stage where our source water (Sandy Hook Bay, New Jersey) is cleaned and ambient pCO_2 is reduced by CO_2 stripping from air and water. The CO_2 treatments are created by diffusing CO_2 into cleaned baywater and distributing controlled CO_2 baywater into specimen exposure containers that are placed in temperature-controlled water baths. The carbonate chemistry was continuously monitored by pH probes and frequently checked against discrete samples evaluated for pH by electrode and spectrophotometer and for DIC by coulometer (full details in Wieczorek et al., unpublished data).

2.3 Husbandry and experimental protocols

Adult summer flounder were collected from local waters of the inner New York Bight (New York and New Jersey), returned to the NOAA-Sandy Hook Laboratory, and acclimated to captivity. Adults were placed in round tanks (2 to 3 m diameter) supplied with local baywater (practical salinity units, PSU, of 24 to 26, pH 7.7 to 7.8), maintained in light and temperature regimes that would lead to autumn spawning conditions, and fed thawed frozen forage fishes three to seven days per week, depending on season. Males ripened spontaneously and females were induced to hydrate oocytes by daily IM injections of LHRH 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 3 females and each female's eggs were mixed with milt pooled from 3 to 5 males, flooded with ultraviolet-sterilized, 0.5 µm filtered baywater to activate the sperm and initiate fertilization, then gently aerated for 2 hr until the first water change. At 2 h post-fertilization, one subset of eggs from each spawning (3 separate maternal sibling groups of offspring) was counted into 3 groups

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of 100 eggs then each group was transferred to one of three CO_2 concentrations in the experimental CO_2-delivery system. These eggs were monitored daily until hatching with removal of dead eggs and hatched larvae. Subsets of hatched larvae per replicate were either anesthetized (MS222) and photographed live for image-based size and shape analyses ($n = 10$), preserved for histopathology ($n = 25$), or preserved as archived samples.

The other subset of eggs from each maternal sibling group was maintained in static, aerated egg incubators with twice daily water change until hatching. At one day after peak hatch nine groups of 500 larvae, drawn from a combination of parental sources, were transferred to larval rearing containers in the experimental CO_2-delivery system resulting in three replicate groups per each of three CO_2 concentrations.

Larvae in the CO_2 experimental system were fed *Nanochloropsis* (Rotigrow) enriched rotifers (*Brachionus plicatilis*) one to two times per day at densities that increased from 0.5 to 20 mL^{-1} with increasing larval size. Advanced larvae (~ 3 week post-hatching) were also offered enriched (DHA Selco) *Artemia* at densities from 0.5 to 5 mL^{-1} that increased with increasing larval size. Larvae were monitored daily and dead larvae were removed until termination of the experiment at 4 week post-hatching.

Subsets of larvae per replicate were sampled on a weekly basis through 4 week (larvae sampled at 0, 14, and 28 d post-hatch are reported here). Sampled larvae ($n =$ 10 per replicate) were anesthetized (MS222), photographed live for later image-based size and shape analyses, and preserved as archived samples. An additional set of larvae ($n = 10$ per replicate) was collected and preserved for histopathological analysis of tissues and organs.

All embryos and larvae were maintained at 21 °C and under a 12 : 12 light : dark photoperiod regime throughout the study. Tank maintenance, food densities, and environmental condition followed protocols previously used at our laboratory.

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2.4 Response variables measured

Although it was expected that the proximate cause of effects on ELS measured here are due to CO_2 levels disrupting the acid-base equilibria at the cellular level, this disruption is likely to be variously expressed at higher levels of biological organization within the individual (e.g., physiological, anatomical, behavioral, and life-historical responses). To some degree, these different types of response variables are inter-related and simply represent different manifestations of the underlying changes caused by the environmental stressors. The response variables were expected to have different sensitivities and time scales but all have the potential to be correlated with the Fisherian fitness of the individual.

The primary response variables in the embryo and pre-feeding larvae sub-experiment reported here are survival rate to hatching and the size and shape morphometrics of hatchlings. Response variables in the feeding sub-experiment are size and shape morphometrics and histopathology analysis of key tissues and organs of larvae.

2.4.1 Survival

The numbers of embryos surviving to hatching was based on daily inspection of the embryos in their CO_2-exposure containers. Hatch-frequency data were converted to proportions surviving within each container (= number hatched/number in start group).

2.4.2 Size and shape of larvae

Size and shape of larvae are viewed here as indices of growth, condition, and developmental stage. The weekly collection of larvae (n = 10) from each replicate population on each sampling day was anesthetized and photographed with a Zeiss Axiocam HRc color digital camera mounted on a Zeiss SteREO Discovery V8 dissecting microscope. Morphological features that were quantified varied with developmental stage of the lar-

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vae but included lengths (total, standard, pre-caudal, flexion, and yolk), depths (total body, musculature at vent, and yolk), oil globule diameter (average of two orthogonal diameters), and developmental markers (mandible size and flexion angle). Morphometric data were extracted from images using Image Tool software.

2.4.3 Histopathology

Tissues and organs were evaluated for normal development and cranio-facial dimensions were quantified from each treatment group for evidence of CO_2 effects. Common ELS toxicities in fish include cranio-facial and skeletal deformities, reduced growth, and cardiac disruption (Barron et al., 2003). Alcian-blue staining of cartilage was used to examine and quantify the extent of cranial-facial deformities, and histopathological analysis was used to discover any abnormalities or lesions in tissues and organs.

The cranial-facial analysis was based on up to 20 fish from each CO_2-exposure and age group. The alcian-blue 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 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. Total length was measured at 1.1 × magnification and cranial-facial structures (mandible, lower jaw, maxilla, and snout length) at 2.4 ×, while noting any abnormal cartilaginous structures or staining characteristics.

The histopathological analyses were based on 8 to 12 fish from each CO_2-exposure and age group. 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 3 to 4

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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 cranial-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.

2.5 Statistical analyses

All hypothesis testing addressed the null hypothesis of no effect of CO_2 levels on the response variables. The statistical analyses applied to these data were predicated on the ANOVA 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., the treatment × replicate mean, median, or summary statistic such as survival) or the interdependence of observations from a group that shared a container was explicitly accounted for by applying multivariate statistical methods (e.g., multivariate ANOVA). The critical value for statistical significance was set at $p = 0.05$. Analyses were conducted with SYSTAT 11 and Sigma Plot 11 software packages.

2.5.1 Survival data

Before analysis, proportions of embryos surviving to hatching were converted to the relative survival proportions for each replicate (= source of eggs) in order to standardized any inter-female differences in survival of their embryos. These relative proportions were transformed for normalization (arcsine square-root) and analyzed as a one-way ANOVA with CO_2 level as the treatment.

2.5.2 Size and shape data

All morphometric analyses were conducted on mean values per replicate within CO_2 level. The statistical analysis of morphometric data was a three-step process. First, due

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to the lack of independence of morphometric variables describing size and shape of the same individual, the morphometric data were reduced by principal component analysis (PCA). Second, the resulting PCA scores of individual fish were reduced to replicate means. Third, the replicate-average principal component (PC) scores were analyzed as an ANOVA or MANOVA depending on whether one or more PC was significant. Follow-up univariate tests for effects, graphical display, and interpretation in the context of the original variables were conducted using ANOVAs.

2.5.3 Histopathology and cranio-facial data

The histopathological responses are reported in a qualitative manor at the tissue level for each CO_2-exposure group and age. The cranial-facial responses were subjected to a one-way ANOVA for no effect of CO_2. In addition, a statistical contrast (two-tailed *t* test) between cranial-facial responses of larvae from the ambient and the highest used CO_2 level was conducted in order to identify effects on responses at the extreme CO_2 environments relative to those at ambient CO_2 conditions.

3 Results

3.1 CO_2 experimental system performance

The embryos and larvae in our experiments experienced elevated CO_2 environments similar to our nominal target values of peak local ambient CO_2 concentrations in nature (based on near-bottom locations and late summer conditions), approximately 2.5 times peak ambient, and approximately 6 times peak ambient. The levels of pCO_2 used and the associated acidities were 775 ppm (pH 7.82), 1860 ppm (pH 7.44), and 4,717 ppm (pH 7.06). Other observed values of key environmental variables are salinities of 24.8 PSU and water temperatures of 21 °C. A full description of system performance for this experiment appears in Wieczorek et al. (unpublished data).

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3.2 CO_2 effects on survival of embryos and pre-feeding larvae

The proportion of fertilized eggs surviving to hatching decreased with increasing CO_2 and water acidity (Fig. 1). The relative survival to hatching was reduced by approximately half as CO_2 increased from ambient CO_2 conditions to 2.5 times ambient, and reduced again by more than half at the highest CO_2 level tested (6 times ambient). This pattern of reduction in relative survival was highly significant and was consistent across all three replicate used despite differences in baseline survival at ambient CO_2. The among-replicate differences in the absolute survival across the three CO_2 levels (i.e., the differences in elevation of lines in Fig. 1) are attributable to the different parental sources used for each replicate.

3.3 Size and shape of larvae

3.3.1 Variance structure of larval morphological variables

The sets of morphological variables used to describe size, shape, and thereby condition of larvae varied with larval age and developmental stage. Eight variables were used for samples taken of 0 and 28 d old larvae (Fig. 2). Three of eight variables (yolk length, yolk depth, and oil globule diameter) were unique to 0 d old larvae and two of eight (flexion length, flexion angle) were unique to age 28 d old larvae. Five variables were shared among larvae of all ages: total length, standard length, pre-caudal body length, total body depth at vent (including finfold or fin integument), and musculature depth at vent. Mandibular length was included in the set of measures of larvae of all ages except 0 d larvae.

PCA confirmed the expectation the sets of morphological variables within individuals at each sample age were not independent and were highly correlated, and provided one or more significant independent linear combinations of the original variables. As expected these new and independent variables (the PCs) captured the variance structure of the original data and offered insights into how larval size and shape changed

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as a function of treatment (CO_2 level) and age. For 0 d and 14 d larvae, only one PC (PC1) was significant (not shown) and accounted for 69 and 85 % of the original among-individual variance, respectively. For 28 d old larvae, two PCs (PC1, PC2) were significant (Fig. 3) and accounted for 77 and 17 % of the variance, respectively (sum of 94 %).

For larvae at each of the three sample ages PC1 is best described as a measure of larval size (PC loadings onto PC1 and PC2 for 28 d old larvae are shown in Fig. 3; values of loadings onto PC1 for all ages are in Fig. 4, inset). Larval lengths (total and standard lengths for all ages, but also including body length and flexion lengths for older larvae) were consistently major contributors to PC1. Measures of body depth of the larvae, especially at older ages, and mandibular 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 measures at all ages. For 0 d larvae, the measures of energy available to the larvae (yolk length and depth, and oil globule diameter) contributed negatively to PC1 indicating an inverse relationship between larval size and energy reserves at hatching. For advanced larvae (28 d old) the significant PC2 reflects developmental events in late larval ontogeny. Here, the extent of flexion, as quantified by flexion angle (Fig. 2), makes a major contribution to PC2, with a secondary contribution to PC2 by the depth of body musculature (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. Within this development axis (PC2), these primary measures of development are inversely related to length, i.e., those fish that are undergoing developmental transition tend to be the shorter fish.

3.3.2 CO_2 effects on larval size and development

Elevated levels of CO_2 and water acidity significantly affected larval size and development (ANOVA on PC1, $p < 0.05$). The pattern of effects changed with larval age, was

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evident in the PC-reduced scores, and is reflected (repeatedly) in the original morphological variables. At hatching, larvae from embryos maintained at ambient CO_2 conditions were smaller in size (length and depth) but had more yolk and larger oil globules than larvae from embryos maintained in higher CO_2 environments. This CO_2 effect is reflected in the PC1 scores for 0 d old larvae (Fig. 4a). This tendency is also evident in any measure of length or depth of 0 d old larvae and in the inverse response in any measure of their energy reserves (e.g., total length and yolk length of 0 d larvae are inversely related, Fig. 5a).

This pattern of smaller larval sizes (lengths, depths, mandibular size) at ambient CO_2 conditions and larger larval sizes in higher CO_2 environments continued through the mid larval period samples at 7 and 14 d post-hatching (data from 14 d larvae shown in Fig. 4b). By 21 d post-hatching (not shown in figures) the size ranking began to shift with larval sizes comparable among all CO_2 environments and a slight trend towards smaller sizes at the highest CO_2 environments. By 28 d post-hatching (~ 80 % through the larval period at the rearing temperature), the larvae at the intermediate CO_2 environment were the largest sizes while larvae at the ambient and highest CO_2 conditions were smaller and comparable in size to each other (Figs. 4c and 5b). Despite a rough size equivalency, larvae in ambient and highest CO_2 conditions were at significantly different developmental stages (Fig. 4d). Larvae from the ambient CO_2 environment were the least advanced of all groups in terms of notochord flexion, those from the mid CO_2 environment were intermediate in their developmental stage at experiment termination, and larvae from the highest CO_2 condition were most advanced, showing signs that metamorphosis was imminent by their deepening bodies and a greater degree of notochord flexion (Fig. 5c).

3.4 Cranio-facial and 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 ≥ 14 d post-hatching (younger larvae could not be reliably prepared for histology). These skeletal

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measures were grouped by age and treatment (CO_2 level) within age and analyzed for CO_2 effects (Table 1). Significant effects of CO_2 were found in the total and maxillary lengths in the 14 d old larvae, and in the lower jaw of 21 d old larvae (i.e., differences via ANOVA in 3 of 15 possible response × age comparisons). A series of statistical contrasts between larvae from the ambient versus the highest CO_2 environment revealed significant differences in 6 of 15 possible contrasts (Table 1). These significant two-group contrasts include the maxilla in 14 d old larvae, all measurements but the snout in 21 d old larvae, and the snout in 28 d old larvae. Many other skeletal measures show a trend in their response to CO_2 levels that varied with age: larvae from ambient CO_2 environments were comparable or smaller in skeletal elements than larvae from elevated CO_2 environments when younger (14 and 21 d old) but had larger elements at older ages (28 d old).

The histopathology evaluations revealed that all larvae examined (age ≥ 14 d post-hatching) had food in their GI tracts (Fig. 6). 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. Larvae from the ambient and intermediate CO_2 environments were similar in appearance; however larvae from the intermediate CO_2 environment tended to express minor liver sinusoid dilation. Larvae from the highest CO_2 environment expressed additional abnormalities (Fig. 6). These abnormalities included minor focal hyperplasia on the epithelium and separation of the trunk muscle bundles. Extensive dilation of the liver sinusoids and central veins was evident which resulted in the liver appearing condensed and organized into rows or patches. Larvae from the highest CO_2 environment also displayed delayed formation of the mandible.

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4 Discussion

4.1 CO_2 effects on embryo survival

A strong effect from 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 (0.4 pH unit reduction) in the environment, and a further reduction by over half for another 2.5 × increase above the intermediate CO_2 levels (a further 0.3 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 (1860 ppm, pH 7.44) is unlikely to be sustainable for this fish stock as this reduction can be expected to be chronic unless summer flounder has the potential to respond via long-term acclimation or natural selection to the CO_2 levels predicted for this and the next century. The pre-spawning adult fish were not conditioned by exposing them to varying CO_2 environments nor was heritability of resistance to elevated CO_2 environments quantified and thus the potential for selective response. The protocol used here of obtaining offspring from different parental sources, retaining family integrity by maintaining offspring in sibling groups, and inspecting the pattern of responses does allow a glimpse at the likelihood of the potential for a selective response. The finding that these separate sibling groups responded nearly identically in their reduced embryo relative 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 warranted on the role of parents to possibly advantage their offspring via either maternal environmental conditioning to elevated CO_2 environments or through heritable variation in these traits. 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).

This degree of reduction in embryo survival in the face of elevated CO_2 environments has not been reported by other authors working on a different and diverse set of marine fish taxa. Munday et al. (2009b) found the survival to hatch of orange clownfish (*Am-*

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phiprion 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 even 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 Northeast Pacific, at pCO_2 levels up to 1933 ppm. In contrast, Baumann et al. (2011) reported a 74 % reduction in survival of young larvae of inland silverside, native to estuaries of the US Atlantic Coast, when maintained at higher pCO_2 levels (1100 ppm) compared to larval survival of 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. (2011) began at ~ 24 h post-fertilization and survival was scored at ~ 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., 2011) is in contrast to our findings. The habitats occupied by a species, particularly its ELS may play a role in their sensitivities to elevated CO_2 environments. Here the habitat and reproductive ecology of summer flounder may render their embryos at relatively high risk to hypercapnia. In particular, summer flounder spawn in relatively stable waters of the continental shelf of the mid-Atlantic states of the USA. Spawning has not been observed directly in situ for summer flounder but flatfish typically spawn off bottom and summer flounder eggs are positively buoyant in local seawater (~ 35 PSU). This combination of relatively stable (CO_2) habitat features, spawning behavior, and pelagic embryos may have limited the range of CO_2 environments experienced by summer flounder and this may be a reason for the restricted resilience in the viability response

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of summer flounder embryos to elevated CO_2 environments compared to the resiliencies reported for other species.

4.2 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 significantly affect recruitment dynamics. Larvae from elevated CO_2 environments were larger at hatching but had smaller yolk and oil globules than larvae from ambient 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 fish (Chambers et al., 1989, unpublished data). Here such a trade-off means that larvae from environments with higher CO_2 would be larger but likely have less time to successfully initiate prey consumption.

All else equal, large size is thought to confer an advantage on those larvae via enhanced prey capture or predator avoidance (Miller et al., 1988). Greater larval lengths and growth rates in CO_2-enriched environments have been observed in inland silverside (Baumann et al., 2011), Atlantic cod (Frommel et al., 2012), and walleye pollock (Hurst et al., 2013). Importantly, and countering the presumed benefits of large size, Frommel et al. (2012) found that longer larval Atlantic cod from high CO_2 environments were in poorer condition and were more likely to display tissue and organ damage than larvae that experienced lower CO_2 environments – a relationship also found here with summer flounder. Using another measure of condition, Franke and Clemmesen (2011) also report that larvae of Atlantic herring from higher CO_2 environments were of lower condition (lower RNA to DNA ratios) than their counterparts in low CO_2 environments.

As shown here, summer flounder larvae from lower CO_2 environments were smaller than those from higher CO_2 environments until at least midway through the larval period. Older larvae displayed a developmental change associated with CO_2 levels that implied a difference in the timing of and likely the size at the metamorphic transition between the larval and juvenile life-stages. Hence, an inverse relationship between CO_2

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levels in the environment and the duration of the larval period can be inferred. Given the growth trajectories and developmental stages at the termination of this experiment (28 d), the larvae at ambient CO_2 levels are expected to be larger than those from the highest CO_2 levels and quite likely larger than those from the intermediate CO_2 level.

Few other OA studies have considered a multivariate morphometric characterization of larvae 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 large-variable characterization of possible CO_2 responses in the early life-history of spiny damselfish (*Acanthochromis polyacanthus*) that were experimentally exposed to a range of pCO_2 levels (450 to 850 ppm) beginning on hatch day. Their characterization included an evaluation of 29 skeletal elements quantified 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 (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 significance in summer flounder (Chambers, unpublished data). Because this species primarily spawns in autumn (September–December), its larvae experience rapidly declining seasonal temperatures. Depending on the timing of spawning, the autumn sea temperatures, and the intrinsic ontogenetic rate, larvae in a summer flounder cohort may metamorphose, ingress, and settle into estuaries before winter, after winter, or both. Importantly, larvae are incapable of metamorphosing at the cool winter water temperatures of this region (December–March) so acceleration or deceleration of developmental timing as

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a consequence of elevated CO_2 levels would amplify the consequences of ingressing in the autumn versus spring.

4.3 CO_2 effects on tissues and organs

The number and severity of cranial-facial malformations in summer flounder larvae increased with levels of CO_2 in the environment. The majority of these differences were seen in older larvae, especially the 21 d old sample which showed significant differences between the highest and ambient CO_2 environments in all cranial-facial measurements except for the snout length. The fewer observed abnormalities in younger larvae (e.g., 14 d old) may be due to their small size (3.4 to 3.7 mm TL) and difficulties in preparing and accurately measuring these larvae. The reduction in frequency of abnormalities in the oldest larvae (28 d old) compared to younger larvae (21 d old) may reflect mortalities of severely impaired larvae before the sample date. High mortality in the embryonic stages was found here in both of the enriched-CO_2 environments and similarly high estimates of mortality – up to 74 % higher in high-CO_2 environments relative to baseline environments – was reported for inland silverside (Bauman et al., 2012). Selective mortality in summer flounder, with the surviving older larvae possibly possessing homeostatic capabilities that accommodate high-CO_2 levels as found in Atlantic cod (Frommel et al., 2012), may also account for the reduction in frequency of developmental anomalies seen here for larvae at advanced ages.

Although the extent of cranial-facial abnormalities lessened in older larvae, the trend towards reduced cranial-facial feature in high-CO_2 environments persisted to the most advanced larvae sampled (28 d old). Indeed, the lengths of all cranial-facial measures in advanced-age larvae tended to be smaller for fish from the highest versus ambient-CO_2 environments. The extent, location, and proximate cause of high-CO_2 induced abnormalities of the cranial-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 as the right eye of a summer flounder larva migrates to the left side of the face (Schreiber, 2000). While

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this morphology has an evolutionary benefit (Schreiber, 2005), the massive change of cranial-facial structures likely renders the remodeling process as one that is highly sensitive to environmental stressors during this period. The metamorphosis of flatfish is influenced proximally by thyroid hormone (TH) which also controls the tolerance of the fish to changes in salinity (Schreiber 2005). 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 was found in summer flounder survival to hatching in high-CO_2 environments. The CO_2 effects on larval size, shape, and developmental staging were more subtle and were revealed through a holistic multivariate approach. Negative effects of a high-CO_2 environment were also evident in larval tissues and cranial-facial features and, like the CO_2 effects expressed in our external phenotypic measures, changed with age and ontogenetic stage. 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 fish species is taking shape in the OA research community. At the very least, this understanding acknowledges the diversity of taxa evaluated, protocols implemented, and patterns of responses revealed in OA studies to date. A prescription for a research strategy that may result in rapid growth of the OA research front was offered above (see Sect. “General approach”) and was used in part in this study. That proposed strategy uses a progression of experiments that first evaluates a diverse array of response variables to a broad range of CO_2 environments, then adds a co-stressor(s) in the next round of experimentation, and ends with experiments on a refined subset of the previously inspected environmental space.

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The refined environmental subspace is defined by boundaries that capture the steepest gradient of change in the response variable(s) found in the earlier experiments.

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

1. Select focal species with life histories and ecologies, and from habitat types that are most likely to be representative of broader species groups. Given the infancy of OA research on marine fishes, the OA community is limited by the small number of taxa studied to date and the likely selection criterion of species for experiments being one of convenience and familiarity rather than a robust strategy of inference.

2. Identify best practices, refine methods where needed, and document all protocols. Due to the international interest that OA has attracted from governmental agencies, international councils, and academicians, the OA research community has available to it recurring opportunities for workshops, symposia, and best practices guidelines (e.g., Riebesell et al., 2010).

3. Provide project metadata to the OA and broader research communities. Inter-study comparisons and retrospective studies will only be as good as the data available for such meta-analyses. To that end, multiple agencies – including NOAA's Ocean Acidification Program – are developing metadata standards. Input for these standards as well as critiques and usage of them are needed in order to maximize their utility for the largest fraction of the OA research community.

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Table 1. Summary statistics (means ± SE) and test of effects for cranial-facial features of summer flounder larvae exposed to different levels of CO_2 (water acidity, pH) in OA experiments. Larvae were collected at three ages (14, 21, and 28 d post-hatching). Significance of test of no effect of CO_2 treatment (one-way ANOVA, "*") and subsequent two-group contrasts (*t* test, "+") between ambient and highest CO_2 (pH) environments are shown within sample age groups and cranial-facial features.

Age (d)	pH	*N*	Total Body Length (mm)	Mandible (mm)	Lower Jaw (mm)	Maxilla (mm)	Snout (mm)
	7.1	18	$3.676 \pm 0.141^{*}$	1.582 ± 0.064	0.420 ± 0.027	$0.108 \pm 0.007^{*+}$	0.439 ± 0.009
14	7.4	18	$3.475 \pm 0.136^{*}$	1.663 ± 0.041	0.373 ± 0.009	$0.093 \pm 0.005^{*}$	0.414 ± 0.009
	7.8	18	$3.700 \pm 0.132^{*}$	1.652 ± 0.042	0.399 ± 0.005	$0.072 \pm 0.004^{*+}$	0.426 ± 0.008
	7.1	18	$4.766 \pm 0.165^{+}$	$2.116 \pm 0.044^{+}$	$0.633 \pm 0.016^{*+}$	$0.175 \pm 0.009^{+}$	0.521 ± 0.006
21	7.4	18	4.530 ± 0.262	2.128 ± 0.051	$0.574 \pm 0.019^{*}$	0.146 ± 0.007	0.518 ± 0.007
	7.8	18	$4.535 \pm 0.283^{+}$	$1.984 \pm 0.048^{+}$	$0.563 \pm 0.019^{*+}$	$0.147 \pm 0.012^{+}$	0.516 ± 0.008
	7.1	18	5.129 ± 0.328	0.425 ± 0.111	0.695 ± 0.024	0.246 ± 0.012	$0.562 \pm 0.011^{+}$
28	7.4	16	5.211 ± 0.353	0.415 ± 0.051	0.708 ± 0.027	0.248 ± 0.012	0.606 ± 0.020
	7.8	19	5.340 ± 0.253	0.450 ± 0.012	0.706 ± 0.022	0.281 ± 0.018	$0.609 \pm 0.017^{+}$

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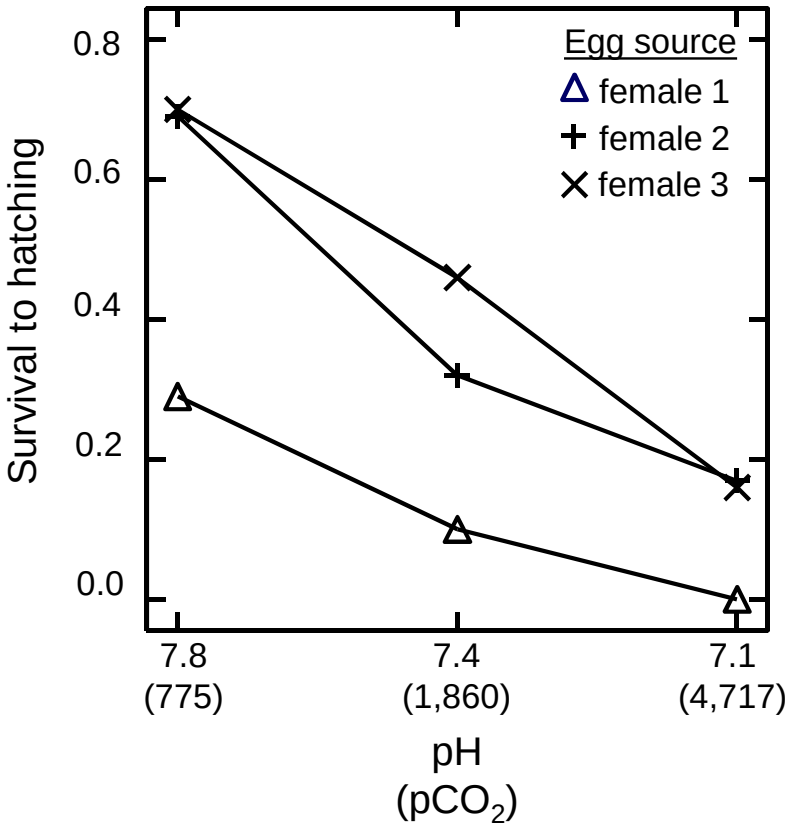

Fig. 1. Proportion of fertilized eggs of summer flounder surviving to hatching at different levels of rearing water CO_2 and pH in OA experiments. Embryos were created from eggs from three females each crossed with milt pooled from three to five males.

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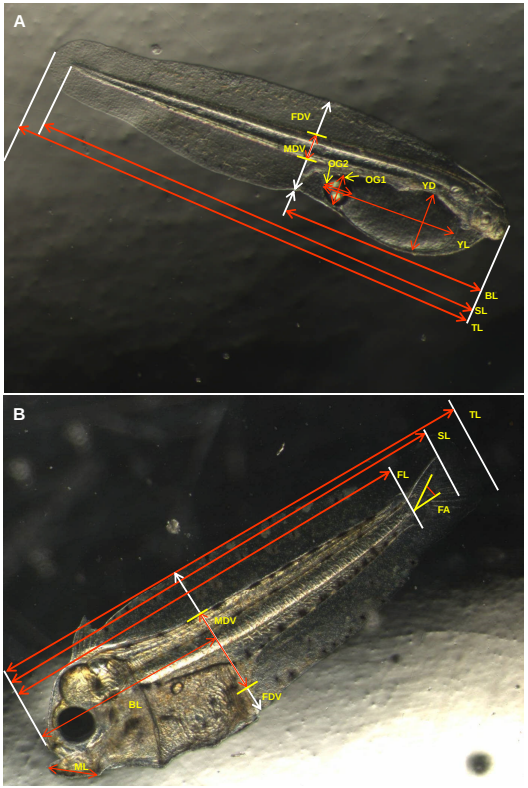

Fig. 2. Morphological variables used to describe size and shape of summer flounder larvae in OA experiment. The variables measured varied with larval age and developmental stage. **(A)** Hatched larva (0 d old). **(B)** Late larva undergoing notochord flexion (28 d old). Abbreviations: Pre-caudal body length (BL), flexion length (FL), standard length (SL), total length (TL), total body depth at vent (including finfold or fin integument) (FDV), musculature depth at vent (MDV), yolk length (YL), yolk depth (YD), oil globule diameters (OG1, 2), mandibular length (ML), flexion angle (FA).

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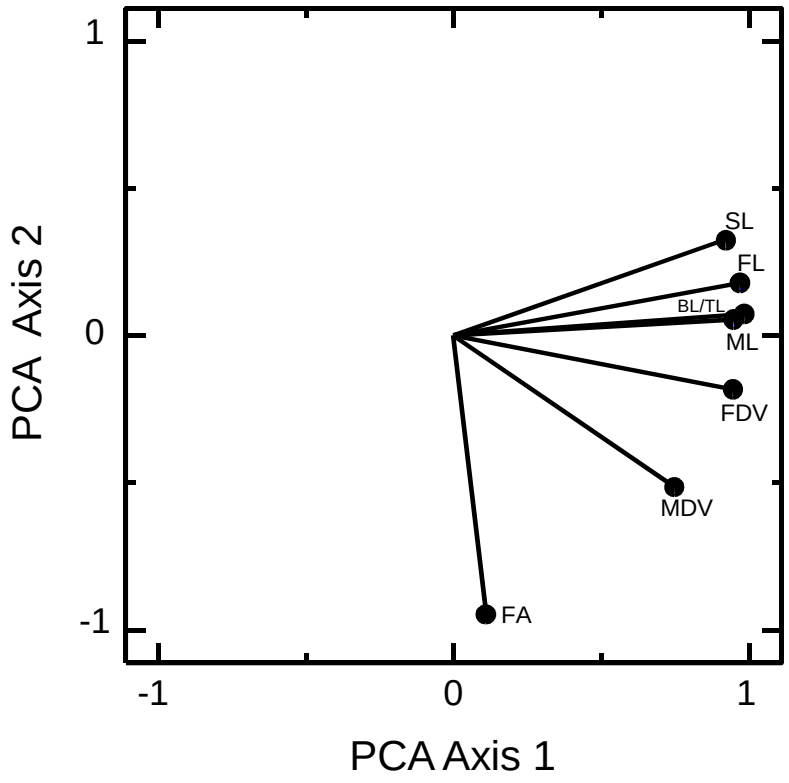

Fig. 3. Variation in loadings of morphological variables measured on late-stage summer flounder larvae (28 d old) from OA experiment. Loadings are calculated by principal component analysis (PCA). PCA axis 1 accounted for 77 % of overall variance and is dominated by measures of fish size (primarily length but also body depth measurements). PCA axis 2 accounted for an additional 17 % of the remaining variance and largely reflects developmental stage (extent of flexion) with contributions by depth of the body musculature. See Fig. 2 for abbreviations.

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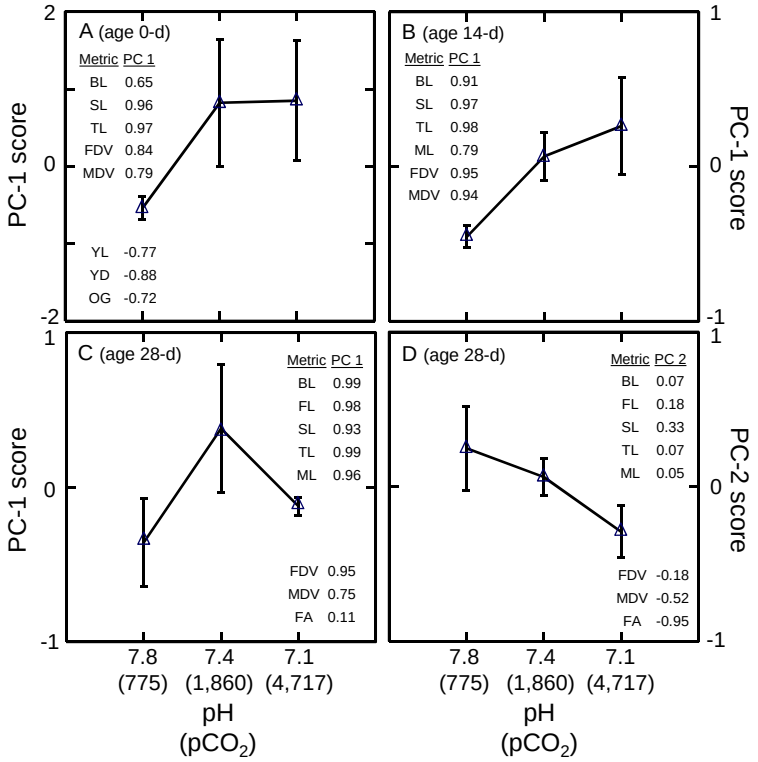

Fig. 4. Responses of summer flounder larvae at different ages to levels of rearing water CO_2 and pH in OA experiments as summarized by principal component (PC) scores. Only significant PC axes are shown. **(A)** PC 1 (0 d old). **(B)** PC 1 (14 d old d). **(C)** PC 1 (28 d old). **(D)** PC 2 (28 d old). The metrics and associated PC weights (inset) represent the contribution of each measured morphometric variable to the composite PC score. Plotted data are means ± SE. See Fig. 2 for abbreviations.

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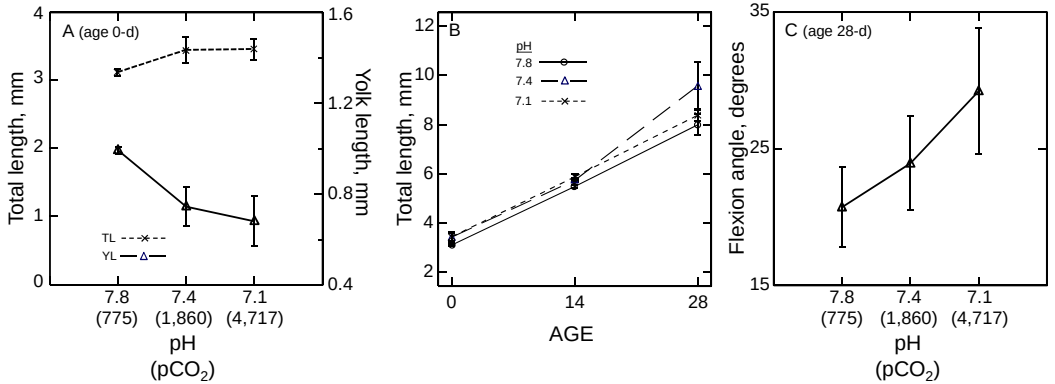

Fig. 5. Responses of summer flounder larvae to different levels of CO_2 (water acidity) in OA experiments. **(A)** Total length (TL) and yolk length (YL) of larvae at hatching (0 d old). **(B)** Total length versus age of larvae for three CO_2 environments. **(C)** Notochord angle at flexion pivot of advanced larvae (28 d old) as an indicator of advanced, pre-metamorphic developmental stage. Flexion angle (see Fig. 2B) increases from zero as metamorphosis is approached. Plotted data are means ± SE.

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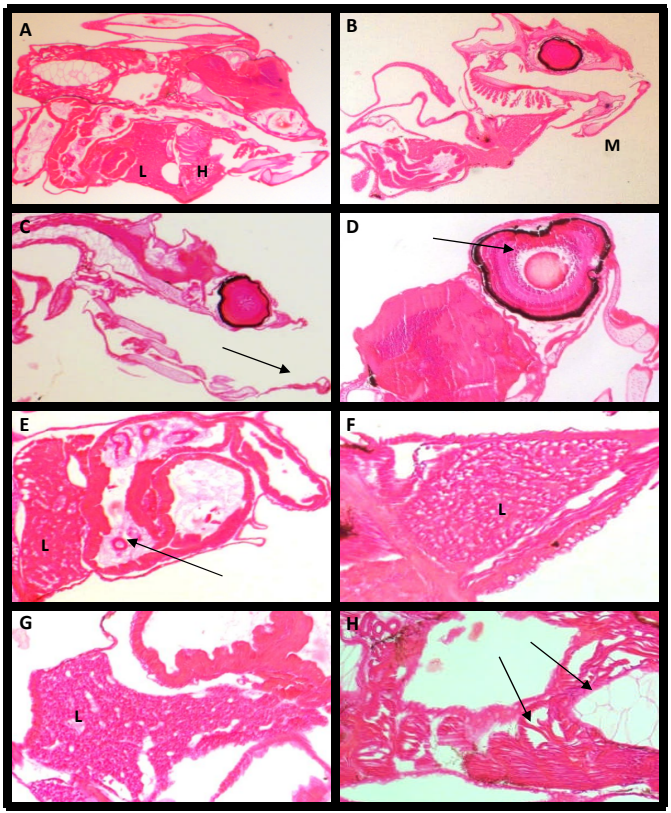

Fig. 6. Images of summer founder (ages 21 to 28 d) tissues stained with hematoxylin and eosin. **(A)** Cranial-facial structures and layout of gall bladder, heart (H), liver (L), gastro-intestinal (GI) tract (5 × magnification). **(B)** Mandible (M) and visualization of the eye location (5 ×). **(C)** Lower jaw hyperplasia (arrow) (5 ×). **(D)** Rods and cones (arrow) (10 ×). **(E)** GI tract including consumed prey (arrow) (5 ×). **(F)** Condensed liver tissue (10 ×). **(G)** More severe liver sinusoid condensation (10 ×). **(H)** Trunk bundle muscle tissue separation (arrow) (5 ×).

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