Title:

Expanding evaluation of ocean acidification responses in a marine gadid: elevated CO_2 impacts development, but not size of larval walleye pollock

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Abstract

Responses of marine populations to climate conditions reflect the integration of a suite of complex and interrelated physiological and behavioral responses at the individual level. Many of these responses are not immediately reflected in changes to survival, but may impact growth or survival at later life stages. Understanding the broad range of impacts of rising CO_2 concentrations on marine fishes is critical to predicting the consequences of ongoing ocean acidification. Walleye pollock (*Gadus chalcogrammus*) support the largest single-species fishery in the world and provide a critical forage base throughout north Pacific ecosystems. Previous studies of high CO_2 effects on early life stages of walleye pollock have suggested a general resiliency in this species, but those studies focused primarily on growth and survival rates. Here we expand on earlier studies with an independent experiment focused on walleye pollock larval development, swimming behavior, and lipid composition from fertilization to 4 weeks post-hatch at ambient (~425 µatm) and elevated (~1230 µatm) CO_2 levels. Consistent with previous observations, size metrics of walleye pollock were generally insensitive to CO_2 treatment. However, 4-week post-hatch larvae had significantly reduced rates of swim bladder inflation. A modest change in the swimming behavior of post-feeding larvae was observed at 4, but not at 2 weeks post-hatch. Although there were no differences in overall lipid levels between CO_2 treatments, the ratio of energy storage lipids (triacylglycerols) to structural membrane lipids (sterols) was lower among larvae reared at high CO_2 levels. Although we observed higher survival to 4 weeks post-hatch among fish reared at high CO_2 levels, the observations of reduced swim bladder inflation rates and changes in lipid cycling suggest the presence of sub-lethal effects of acidification that may carry over and manifest in later life stages. These observations support the continued need to evaluate the impacts of ocean acidification on marine fishes across a wide range of traits and life stages with replicated, independent experiments.

KEYWORDS

Behavior, fatty acids, growth rate, lipids, ocean acidification, swim bladder

Introduction

Changing climate conditions are predicted to have profound impacts on the structure and dynamics of marine ecosystems, including impacting species supporting critical wild capture fisheries. Rising temperatures have already led to pole-ward changes in the distribution of numerous species (Perry et al., 2005; Campana et al., 2020; Yasumiishi et al., 2020). Anthropogenically elevated levels of atmospheric

carbon dioxide (CO_2) have directly led to increased CO_2 levels and reduced pH of ocean waters, referred to as "ocean acidification" (OA, Caldeira and Wickett, 2005). The degree of OA is expected to vary regionally due to local factors such as temperature, mixing rates, and freshwater input, with global models RCP 6.0 and 8.5 predicting more than a doubling of surface ocean CO_2 levels by the end of the 21st century (Ciais et al., 2013). Significant concern has arisen that OA will disrupt the functioning of marine ecosystems and reduce the productivity of important fishery resources (Cooley and Doney 2009; Denman et al., 2011) and the communities that rely upon those resources, both economically and nutritionally (Mathis et al., 2015; Ekstrom et al., 2015). The high-latitude seas of the north Pacific Ocean are of particular concern because they are predicted to be acutely affected by both acidification and continued warming (Fabry et al., 2009; Mathis et al., 2011; Cheung and Frölicher, 2020).

Experimental studies on marine organisms have demonstrated a range of effects from elevated CO_2 and reduced pH (Fabry et al., 2008; Kroeker et al., 2010; Ashur et al., 2017). In general, fishes are expected to be more resilient to some of the direct physiological effects of OA than invertebrates with carbonate exoskeletons (Pörtner et al., 2004; Melzner et al., 2009). However, a number of studies have shown sensitivity to elevated CO_2 levels in the growth and survival rates in the embryonic and larval stages of marine fishes (Baumann et al., 2012; Hurst et al., 2016; Murray et al., 2019), but these patterns are not consistent across species, with other studies showing no effects on growth and survival (Munday et al., 2011; Bignami et al., 2013; Davis et al., 2016).

Documented effects of elevated CO_2 in larval fishes include behavioral changes (Cripps et al., 2011; Forsgren et al., 2013; Williams et al., 2019), hyper-calcification of otoliths (Checkley et al., 2009; Bignami et al., 2013; Holmberg et al., 2019), developmental anomalies (Frommel et al., 2014; Pimentel et al., 2014; Chambers et al., 2014) and changes in respiration, protein and lipid synthesis (Franke & Clemmesen 2011, Cattano et al., 2016, Murray et al., 2017). Several studies have found evidence of CO_2 effects on lipid metabolism in larval fishes resulting in changes to overall lipid levels, as well as lipid classes (Hurst et al., 2019) and fatty acid proportions (Díaz-Gil et al., 2015). Changes in lipid metabolism in response to elevated CO_2 appear to be species-specific and are dependent on ontogeny and the degree of acidification (Frommel et al., 2012, Frommel et al., 2019, Hurst et al., 2019, Frommel et al., 2020). Importantly, findings of physiological and behavioral impacts of high CO_2 on early life stages of fishes suggests the potential for carry-over effects on later life stages. These observations also suggest that a focus on growth responses may underestimate the potential impacts of OA and that examination across a suite of traits is required to fully understand the potential impacts of OA on fishery species (Cattano et al., 2018).

Walleye pollock support the largest single-species fishery in the world, with U.S. harvests in the Bering Sea and Gulf of Alaska averaging 1.4 million metric tons over the past 10 years (Fissel et al.,

2019). Walleye pollock also serve as a primary forage species for marine mammals and other commercially important groundfishes (Gaichas et al., 2015). Improved understanding of the responses of walleye pollock to environmental and climate drivers is critical to forecasting the long-term ecological and socio-economic dynamics of the region (Holsman et al., 2019). Two previous studies examining the sensitivity of walleye pollock early life stages (Hurst et al., 2012 & 2013) suggested a general resiliency to elevated CO_2 levels, but those conclusions were based primarily upon assessment of survival, growth and morphometric-based condition metrics. Validating initial findings and providing broad multi-trait evaluations are critical to robust characterizations OA-sensitivity (Baumann et al., 2018; Murray and Baumann 2020). Here we supplement initial findings for walleye pollock larvae with examination of additional response metrics based on independent laboratory experimentation and analysis. These findings provide additional support for previously reported responses of growth and morphometric condition and provide the first assessments of potential high CO_2 effects on larval behavior, lipid composition, and morphological development in this species.

Materials and methods

CO_2 manipulation and monitoring

Walleye pollock eggs and larvae were reared at ambient and elevated CO_2 levels using an automated CO_2 regulation system at the Alaska Fisheries Science Center laboratory in Newport, Oregon used in previous work with walleye pollock (Hurst et al., 2013) and Pacific cod (*Gadus macrocephalus*; Hurst et al., 2019). For egg incubations, the salinity of local seawater was raised to 34 PSU with the addition of an artificial sea salt mixture (this maintained high buoyancy of the eggs during incubation) in two 180-L reservoirs maintained at 6°C. The CO_2 level in one of these tanks was elevated through automated bubbling of CO_2 gas controlled by a pH probe (Honeywell Durafet III). This temperature-, salinity-, and CO_2-conditioned seawater was used for daily water changes during the egg-incubation phase of the experiment.

A second, identical CO_2 regulation system was used to control temperature and CO_2 levels of a flow-through culture system used during the larval rearing phase of the experiment. In this system, temperature-controlled seawater was introduced into two 400-L, aerated conditioning tanks. Into one of these, CO_2 was bubbled as needed based on continuous measurements of the pH probe. Water from these conditioning tanks was pumped to elevated header tanks which gravity-fed 100-L larval rearing tanks at a rate of 500 mL/min.

The target pH of 7.6 for the elevated CO_2 treatment corresponds to a CO_2 level of ~ 1200 µatm chosen to reflect the upper end of predicted conditions in the Bering Sea and Gulf of Alaska in the next 100 years (Mathis et al., 2015). A separate pH probe (Durafet III) was used to make daily measurements of pH in egg incubation jars and larval rearing tanks throughout the experimental period. In addition,

characterization of the carbonate system in each treatment was based on analysis of twice-weekly water samples drawn from the water supply for each treatment. Oxygen levels measured periodically throughout the experiment (Yellow Springs International Model 85 meter) were consistently above 8.0 mg/L. Water samples were fixed with mercuric chloride and analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA) using an AIRICA (Automated InfraRed Inorganic Carbon Analyzer) and VINDTA 3C (Versatile Instrument for the Determination of Total dissolved inorganic carbon and Alkalinity) at the Ocean Acidification Research Center at the University of Alaska at Fairbanks. The AIRICA and VINDTA 3C instruments were calibrated using Certified Reference Materials (CRMs) from the Dickson Laboratory at the Scripps Institute of Oceanography and a daily correction was applied. Mean deviation of measurements from CRM values were ± 1.39 $\mu mol \cdot kg^{-1}$ for DIC and ± 1.93 $\mu mol \cdot kg^{-1}$ for TA. These measurements were used to calculate the pH and CO_2 conditions during the experiment (Table 1) based on the dissociation constants of Dickson and Millero (1987).

Egg Incubation and larval culture

Experimental procedures were generally consistent with those of Hurst et al. (2013). A broodstock of ~ 35 adult walleye pollock were maintained in a 10-m diameter tank under seasonally varying temperature and photoperiod at ambient CO_2 levels (400-500 µatm during the spawning period). Broodstock fish were allowed to spawn naturally in the tank and fertilized eggs were collected with a plankton net from the surface outflow from the tank. Although the specific parentage of the eggs was not known, to initiate the experiment, we selected a large batch of eggs to ensure at least 2-3 females contributed to the egg batch. Approximately 1500 eggs were introduced to each of eight 3.8-L glass incubation jars. Incubation jars were maintained in a temperature controlled room at 6°C under a 12:12 light:dark photoperiod. Each day during the incubation period 50% of the water in each jar was removed, and the jar was refilled with water of the same temperature and CO_2 level.

At 9 days post-fertilization (DPF), the eggs from each incubation jar were transferred to separate (n=8)100-L, flow-through larval rearing tanks. Temperature and CO_2 levels were maintained at the same levels as during egg incubation, but salinity was allowed to vary naturally (29 PSU at the time of transfer, rising over following 10 days and stabilizing at ~ 31.5 for the remainder of the experimental period). Egg hatching occurred between 11 and 14 DPF (13 DPF functionally defined as the day of hatch). Prey was introduced to rearing tanks beginning at 2 days post hatch (DPH). Fish were fed rotifers (*Brachionus plicatilis*) cultured on a marine microalgal blend (RotiGrow® OneStep, Reed Mariculture, Campbell, CA) and were then enriched with Algamac® 3050 (Aquafauna, Hawthorne, CA) prior to larval feeding. Rotifers were supplied to larval fish tanks at densities of 5 prey/mL twice daily.

Larval sampling

Fish were sampled from the rearing tanks for biological measurements after 2 and 4 weeks of feeding (corresponding to 17 and 31 DPH; 33 and 47 DPF). At each sampling date, three independent samples from each tank were preserved for analysis: 15 fish were used for size measurements; 50 fish were preserved for histological analysis; and 50 fish were used for lipid class and fatty acid analysis. Following sampling at 4 weeks, all remaining fish were counted to determine estimated survival rates. Fish sampled for size measurements were individually photographed under a dissecting microscope (20x) for determination of standard length (SL), body depth (BD, width of myotome band at the anus), and eye diameter (ED). Photographed fish were then pooled into 3 samples of 5 fish each for determination of mean dry weight (DW). Body depth condition factor (K_{BD}) was calculated as the deviation (*100) from a 2^{nd}-order polynomial equation describing the relationship between SL and BD across all fish sampled during the experiment. Dry weight condition factor (K_{DW}) was similarly calculated as the deviation (*100) from the overall SL-DW relationship. Fish body size and condition metrics were analyzed on each sampling date using linear mixed effect model with replicate tank as a random factor.

The patterns of survival during the experiment were based on the initial egg stocking density, estimated counts of daily egg mortality (eggs that had sunk to the bottom of the incubation container were assumed to have died and were removed during daily water changes), and the count of larvae remaining at the end of the experiment. These were used to estimate the number of fish in three "fate" categories: 1) mortalities during the egg phase (in the incubation jars), 2) mortalities during the larval phase (after transfer to flow-through tanks), and 3) survivors (alive at the 4-week sampling period). While the number of larvae surviving at the end of the experiment was precisely counted, the initial number of eggs was based on a volumetric sample. As such, survival patterns should be considered a general reflection of relative differences in survival among treatments. The number of surviving larvae and estimated survival fraction (arcsine transformed) in each tank during the egg and larval phases of the experiment were examined with 1-way ANOVA.

Larval activity patterns

Early stage, larval walleye pollock are very sensitive to handling which precluded behavioral trials outside the culture tanks (*sensu* Maneja et al., 2013; Hurst et al., 2019). Therefore, post-feeding routine activity levels of larval walleye pollock were examined in the culture tanks. Two of the four replicate tanks in each CO_2 treatment were selected for observations on 3 consecutive days starting when the fish were 2 and 4 weeks old. Starting at 30 min after prey introduction, an individual larvae was selected for observation and the movements of this fish recorded for 1 min. During the 1-min observation period, the observer recorded the number of distinct movements (characterized as forward swimming, turning, or

striking at a prey item; Colton and Hurst 2010). To minimize the effects of tank walls or the surface to effect behavior patterns, fish selected for observation were ~ 7-25 cm below the water surface and > 7 cm from the tank wall. At the end of the 1-min observation period, another fish was identified and observed until 5 fish had been observed. All activity scoring was conducted by the same person.

For each observation, the counts of swims, turns, and feeding strikes were summed for an index of total activity which was analyzed with a linear mixed effects model with fish age and CO_2 treatment as main effects and tank as a random effect. Counts of feeding strikes were examined with a model based on a Poisson distribution. Finally, the frequency of turns was analyzed with a mixed effects model that also included the frequency of swims as a linear covariate for evidence of a CO_2 effect on pattern of activity.

Pathologic analyses

On each sampling date, fish for histological analyses were euthanized with an overdose of anaesthetic (MS-222) and preserved in Dietrich's Fixative. After 48 h, samples were transferred to 50% ethanol for storage prior to analysis. An initial histopathologic screening on whole larvae was conducted to identify the range and general frequency of abnormalities in all organs. Sub-samples of larvae (ages 10-31 DPH) from this experiment (n=43) and similar culturing of an independent set of fish reared from the eggs of wild-caught walleye pollock (n=131) was performed. Groups of 28 fish were arranged in agar blocks to prepare sagittal sections as described by Sanders et al. (2014). Agar blocks were placed in 10% buffered formalin and processed for histology with 3 sagittal sections/block to optimize visualization of the organs of most of the fish. The first section was taken at eye level, and the two remaining spaced 40 µm apart. Sections were cut at 5 µm and stained with hematoxylin and eosin.

In this initial screening of 174 fish, we discovered that the only prevalent and significant histological change that occurred in several fish among the samples was presence or absence swim bladder inflation (Fig. 1). Based on these preliminary screening results, we focused subsequent analysis on the presence/absence of inflated swim bladders in fish from the 4 week post-hatch sampling point. Even with multiple cuts and orientating fish in agar blocks, proper visualization of swim bladders was not achieved with several fish. Therefore, we use whole-larvae microscopy to better assess swim bladder development. For this analysis we examined an independent sample of 25 fish from each experimental tank using wet mounts of whole fish. Fish were examined at 25x and 50x with a compound microscope. Digital photographs were used to measure standard length of each fish and swim bladder size (length of longest axis) when present.

Because swim bladder inflation represents an ontogenetic/developmental milestone, the likelihood of fish having an inflated swim bladder was assumed to be affected by body size. Therefore, we used a logistic regression of swim bladder inflation (presence/absence) with body size and CO_2

treatment as main effects and tank replicate as a random factor. For fishes with an inflated swim bladder, we examined the effect of CO_2 treatment on the relationship between fish size and swim bladder size with ANCOVA.

Lipid analyses

Following 2 and 4 weeks of larval culture, 50 ± 3 larvae were sampled from each experimental tank which resulted in a total lipid extract strength of ~500 µg at 2 weeks and ~1000 µg at 4 weeks. Larvae were anesthetized with MS-222 and pipetted onto pre-combusted glass fiber filters (Whatman GF/C). Filters were immediately submerged in 2 ml of chloroform under a blanket of nitrogen and stored at –20°C until extraction, within 6 months of sampling.

Lipids were extracted according to Parrish (1987) using a modified Folch procedure (Folch et al., 1956). Total lipids and lipid classes were determined by thin layer chromatography with flame ionization detection (TLC-FID) using a MARK VI Iatroscan (Iatron Laboratories) as described by Lu et al. (2008) and Copeman et al. (2017). Briefly, extracts of lipid were micropipetted onto duplicate silica-gel-coated Chromarods and developed in a three-stage solvent development system which resulted in the separation of five identifiable lipid classes (wax esters, triacylglycerols, free fatty acids, sterols, and polar lipids). After the last development, rods were scanned using Peak Simple software (ver. 3.67, SRI Inc.) and the signal, detected in millivolts, was quantified using lipid class-specific calibration curves. Standards for each lipid class were purchased from Sigma (St Louis, MO, USA) and a gadid-specific liver based triacylglycerol (TAG) standard was purified using column chromatography following the methods of Miller et al. (1998) and detailed in Copeman et al. (2017). Walleye pollock lipids were expressed both in absolute (lipid per dry weight, $mg \cdot g^{-1}$) and relative amounts (% of total lipids). The ratio of triacylglycerols (TAG, energy storage lipids) to sterols (ST, structural membrane lipids) was used as a lipid condition factor (TAG/ST).

Lipid extracts were derivatized to form fatty acid methyl esters (FAME) using acid transesterification with H_2SO_4 in MeOH (Hilditch Reagent, Budge et al., 2006). Before derivatization, tricosanoic acid methyl ester (23:0) was added to each lipid extract at ~10% of the estimated total FAME mass. Derivatives were run on an HP 7890 GC FID equipped with an autosampler and a DB wax GC column (Agilent Technologies, Inc., USA). The column was 30 m in length, with a film thickness of 0.25 µm, and an internal diameter of 0.25 mm. The column temperature began at 65°C, held for 0.5 min, and then raised to 195°C (40°C/min), held for 15 min and then increased again (2 $°C \cdot min^{-1}$) to a final temperature of 220°C and held for 1 min. The hydrogen carrier gas flow was 2 $ml \cdot min^{-1}$ and the injector temperature was set at 250°C, with the detector temperature held constant at 250°C throughout the 31-min run. Peaks were identified using retention times based on standards purchased from Supelco (37

component FAME, BAME, PUFA 1, PUFA 3). Nu-Check Prep GLC 487 quantitative FA mixed standard was used to develop correction factors for individual FAs. Chromatograms were integrated using Chem Station (version A.01.02, Agilent).

A two-way generalized linear model was used to look at the effect of age (2 versus 4 weeks) and CO_2 treatment on tank means of total lipids and lipid class parameters. When a significant interaction (week*CO_2) was identified, we used Tukey's method to determine the significance of all pairwise comparisons (95% confidence intervals).

Fatty acids >2% in all samples as well as the proportions of triacylglycerols (%TAG), phospholipids (%PL), sterols (%ST), and free fatty acids (%FFA) were included in a multivariate analysis using PRIMER *v*7 (Primer-E Ltd) with the add-on PERMANOVA package. Lipid data were square-root transformed and were then used to calculate a triangular Bray-Curtis similarity matrix between each pair of samples. Non-metric multidimensional scaling (nMDS), was used to illustrate the effect of age and CO_2 treatment on the lipid composition of 2- and 4-week old larvae. A two-way Permutational MANOVA (PERMANOVA) was conducted using the Bray-Curtis matrix of similarities to examine both main and interactive effects of sampling week and CO_2 on walleye pollock lipid composition. Pairwise comparisons were utilized to examine the interactive effects of sample week and CO_2 on the lipid composition of the larvae.

Results

Fish size and survival

Across the experiment there was little effect of CO_2 treatment on body size metrics (Table 2). At 2 weeks of age, there was no significant differences in SL, BD, ED, or K_{BD} between the low and high CO_2 treatments (all $p > 0.10$). However, fish reared at high CO_2 had higher DW ($p = 0.046$) and K_{DW} ($p = 0.011$) than the fish reared at ambient CO_2 levels. At 4 weeks of age, there were no significant differences between CO_2 treatments in any of the body size metrics (all $p > 0.10$). There was no indication of density-dependent growth as larval size (SL) at 4 weeks was not correlated with the number of surviving larvae ($r = 0.372$, $p = 0.156$).

Of the estimated 1500 eggs used to initiate the experiment, the number of surviving larvae remaining at 4-weeks post-hatch ranged from 118 to 363 (Fig. 2). There were more surviving larvae in the high CO_2 treatment tanks than the ambientCO_2 tanks (mean = 299 v. 156; $F_{1,6} = 11.5$, $p = 0.015$). The effect of CO_2 on survival appears to have occurred during the hatching and/or post-hatch phases. Estimated survival of eggs between fertilization and transfer to flow-through tanks at 9 DPF averaged 76.9% (daily mortality rate 2.94%) and did not differ among CO_2 treatments ($F_{1,6} = 0.160$, $p = 0.703$). Conversely, estimated survival following transfer to the larval rearing tanks (2 d prior to initial hatch)

until the end of the experiment averaged 19.9% (daily mortality rate 4.86%) and was significantly higher in the high CO_2 treatment than the ambientCO_2 treatment ($F_{1,6} = 8.177$, $p = 0.029$).

Activity patterns

There was no apparent effect of CO_2 treatment on post-feeding activity level of larval walleye pollock. Total activity level was higher among 4-week than 2-week old fish ($p < 0.01$), but was not affected by CO_2 level or the interaction with age ($p > 0.60$). Feeding strikes 30 min after prey introduction ranged from 0-10 (mean 1.07) and were not affected by fish age or CO_2 treatment ($p > 0.30$). The type of movement expressed by the fish was analyzed by examining the frequency of turns including the frequency of swims as a covariate as there was a negative overall relationship between the numbers of swims and turns ($p = 0.021$; Fig. 3). At 2 weeks old, there was no effect of CO_2 on type of activity ($p = 0.701$). However, at 4 weeks old, there was a trend toward larvae in the high CO_2 treatment exhibiting a greater number of turns relative to swims ($p = 0.083$) suggesting a change in orientation or routine swim path trajectory.

Pathologic analyses

The only histological change observed in baseline screening of walleye pollock larvae by histology was variability in inflation of the swim bladder. Inflated swim bladders were characterized by the presence of a large, oval vacuole lined by epithelium between the notochord and intestine (Fig. 1a, c). In contrast, in fish with uninflated swim bladders, this space was occupied by extensive epithelial tissue (Fig. 1b, d). With whole, intact fish examinations (Fig. 1c, e), as with histology, the inflated swim bladders were distinguished by the presence of a prominent vacuole, ranging in size based on long dimension from 0.15 to 0.69 mm, with an average of 0.41 mm. The swim bladder of fish with uninflated swim bladders had minimal lumen spaces and prominent, folded cuboidal to columnar epithelium (Fig. 1d). Because identification and size measurement of the swim bladder could be influenced by the precise orientation of the histological section, swim bladder inflation rates and measurements were based on examination of whole fish mounts.

Exposure to elevated CO_2 levels resulted in significantly reduced rates of swim bladder inflation in larval walleye pollock; inflation rates evaluated at 4 weeks were 75% in fish reared at ambient CO_2 levels compared to 58% in fish reared at high CO_2 levels. Logistic regression confirmed influence of body size ($p < 0.001$) and CO_2 level ($p = 0.003$) on swim bladder inflation rates (Fig. 4a). Within the ambient CO_2 treatment, one replicate tank had lower inflation rates than 2 of the other replicate tanks (logistic regression tank effect $p < 0.05$), but this was in the tank with the smallest overall body sizes (reflecting body size effect on inflation rate). Conversely, there was no difference in inflation pattern among the high

333 CO_2 tanks (all pairwise contrasts $p > 0.30$). Among fish with inflated swim bladders, swim bladder size
334 was positively related to fish body size (ANCOVA SL covariate $p < 0.001$) but there was no effect of CO_2
335 treatment ($p = 0.6710$; Fig. 4b).
336
337 **Lipid composition**
338 There was no significant effect of CO_2 on total lipid per larvae (Fig. 5a) at week 2 or 4, but there was a
339 significant increase in total lipids (μg) from early to later sampling dates ($F_{1,12} = 98.74$, $p < 0.0001$). In
340 contrast, lipid density per DWT did not show a significant effect of either week of sampling (2 to 4) or
341 CO_2 treatment (Table 3) indicating that energetic reserves were being allocated towards growth rather
342 than increased energy storage (triacylglycerols, TAG). The lipid-based condition ratio of
343 triacylglycerols:sterols (TAG:ST) showed a significant interactive effect of age and CO_2 treatment (Fig.
344 5b, $F_{1,12} = 17.29$, $p = 0.001$), with no difference in larval condition at week 2 but a higher TAG/ST ratio
345 under ambient than high CO_2 conditions at week 4 (Fig. 5b).
346 Closer examination of individual lipid classes showed a significant effect of CO_2 on sterols in
347 larval walleye pollock. Significantly higher amounts of sterols per larvae (primarily cholesterol) were
348 found in larvae from the high CO_2 treatment compared to those reared at ambient (Fig. 5d, $F_{1,12} = 9.59$, p
349 < 0.009). The storage lipid class, TAG, reflected a significant interactive effect of week of sampling and
350 CO_2 level on larval storage (Fig. 5c, $F_{1,12} = 10.21$, $p < 0.008$). At week 2 there was no significant
351 difference between the CO_2 treatments while at week 4, larvae in the ambient CO_2 treatment had elevated
352 TAG compared to those in the high CO_2 treatment. Both the reduced TAG and elevated ST in larvae from
353 the high CO_2 treatment contributed to the significantly lower lipid-based condition factor (TAG:ST) at
354 week 4 (Fig. 5b).
355 Multivariate analyses of the effect of CO_2 and week of sampling on larval lipid parameters using
356 PERMANOVA showed a significant interactive effect of week of sampling and CO_2 (pseudo$F_{1,12}$=16.41,
357 p=0.001, Fig. 6). Pairwise comparisons indicated that larvae differed significantly between CO_2
358 treatments at week 4 (p=0.03) but not at week 2 (p=0.08). Examination of spatial distribution of samples
359 based on their lipid composition using *n*MDS showed a temporal separation of samples within the high
360 CO_2 treatment along the vertical axis associated with differences in lipid class composition. Larvae at
361 week 2 had higher proportions of TAG while at week 4 they had higher proportions of ST and PL. This is
362 in agreement with the univariate analyses of the TAG:ST ratio (Fig. 5b) that showed a significant decline
363 in the lipid-based condition factor in high CO_2 larvae at week 4. The separation of larvae in the ambient
364 CO_2 treatment from week 2 to week 4 along the horizontal axis represented the accumulation of lipids
365 from their prey (enriched rotifers; Table 3) into larval tissues after 4 weeks. Specific fatty acids elevated
366 in enriched rotifers such as 22:5n-6 and 18:2n-6, were also elevated in week 4 larvae from the ambient

CO_2 treatment. This elevation was to a greater degree than that observed in the high CO_2 treatment at week 4 and is indicative of increased assimilation of dietary lipids from enriched rotifers into walleye pollock larval in the ambientCO_2 treatment compared to the high CO_2 treatment.

Discussion

Expanding upon previously published evaluations of OA-sensitivity in walleye pollock larvae (Hurst et al., 2013), we present independent experimental data on additional aspects of potential sensitivity. These observations provide additional support for some aspects of robustness of walleye pollock to elevated CO_2 (in terms of growth rates and early survival rates). However, we also identified aspects of sensitivity (reduced rates of swim bladder inflation and alteration of lipid metabolism dynamics) that were not examined in the previous study. As observed in Hurst et al. (2013), there were no consistent effects of exposure to elevated CO_2 on size and condition metrics of walleye pollock larvae. At 2 weeks post-hatch, larvae in the high CO_2 treatment were heavier than fish from the ambient CO_2 treatment (also reflected in higher K_{DW} values). However, by 4 weeks, there were no significant differences between treatments in any of the size metrics. This may reflect CO_2-specific sensitivity or a more generalized response in the growth-development patterns (Stiasny et al., 2019; Murray and Baumann 2020), as a similar shift was observed in the TAG:ST condition ratio.

Morphological effects of OA

Morphological and histological analyses have been applied in evaluating the potential impacts of high CO_2 on the development of larval fishes. These have examined the occurrence of malformed skeletal elements (Pimentel et al., 2014; Crespel et al., 2017) or other morphological features (Stiasny et al., 2017). Studies that screened for developmental anomalies across a range of organs based on histological preparations (Frommel et al., 2012; Chambers et al., 2014) have identified a variety of pathologic changes (most notably vacuolation in the eye, kidney, and liver) associated with high CO_2 exposure in larvae of a diversity of species (Frommel et al., 2012, 2014 & 2016). We also initially used this approach as it allows for the potential identification of developmental anomalies in all organs when the entire fish is processed. However, there is an underlying subjectivity with interpretations of histological changes and linking them to specific causes, potentially resulting in considerable differences in interpretations between pathologists (Wolf et al., 2015). Moreover, variation in pathologic changes between studies using the same toxicant may be caused by subtle differences in experimental design or fish age. All of this may lead to difficulties in validating observed patterns within a species as well as discriminating methodological differences from species-specific sensitivity when comparing across studies (e.g., Frommel et al., 2014 & 2019). The implications of observed pathology may be ambiguous; for example, although vacuolation of the liver

401 may be a true toxicopathic change, this diagnosis should be applied with caution as increases in
402 hepatocyte vacuoles, either lipid or glycogen, is often correlated with differences in feeding rates and
403 nutritional state (Boorman et al., 1997; Wolf and Wolfe 2005). Finally, it should be considered that
404 histologic changes in larval fish in toxicant exposures studies could be non-specific perimortem events
405 that occur while the affected fish is dying.
406 In this study, we performed an initial histologic screening of walleye pollock larvae from this
407 experiment and an independently cultured group of fish to evaluate the types and frequency of pathologic
408 changes without any a priori endpoints. While occurrences of abnormal appearance in gills, etc., were
409 exceedingly rare (<5%), we found a large number of fish without an inflated swim bladder. Unlike other
410 developmental traits, the presence of an inflated swim bladder is readily observable and does not require
411 subjective assessment of organ appearance. In fact, scoring of swim bladder inflation was more reliable in
412 whole body mounts improving processing efficiency. Interestingly, in fish with an inflated swim bladder,
413 there was no effect of CO_2 level on the size of the swim bladder, resulting in a clear dichotomous
414 indicator of this developmental indicator. We suggest that examination of swim bladder inflation rates
415 could be a valuable and efficient response variable in examining the effect of CO_2 and other stressors on
416 larval fishes.
417 We found a significant negative effect of high CO_2 on swim bladder inflation rate, representing a
418 sub-lethal effect of high CO_2 in larval walleye pollock. Inflation of the swim bladder involves a
419 combination of morphological organ development and the action of moving to the water surface and
420 "gulping" air which passes through the pneumatic duct, filling the swim bladder (the pneumatic duct is
421 lost later in larval development of gadids and other physoclistus species; Woolley and Qin, 2010). As
422 such, reduced or delayed swim bladder inflation in larval walleye pollock exposed to elevated CO_2 levels
423 could reflect either a morphological or behavioral impairment. While fish can survive without an inflated
424 swim bladder for some time, such fish have significantly higher metabolic rates, can have difficulty
425 maintaining orientation and less successful feeding (Czesny et al., 2005; Schwebel et al., 2018),
426 ultimately resulting in reduced survival rates (Woolley and Qin 2010). Because the food-replete and
427 predator-free laboratory culture conditions may partially mitigate these effects, additional work should
428 explore the long-term consequences ("carry-over effects") of the range of observed CO_2-induced
429 developmental errors on early life stages.
430
431 **Larval behavior**
432 A number of studies have suggested that exposure to elevated CO_2 levels alters the behavioral
433 characteristics of larval and juvenile marine fishes (Clements and Hunt, 2015; Nagelkerken and Munday,
434 2016). These studies have examined a range of behaviors which can be generally classified as "routine"

or "response to stimulus." In the former, traits such as activity level, movement speed, and turn rate are examined either in the rearing vessels or following transfer to specific observation arenas. These studies have generally found only minimal or no effect of elevated CO_2 on behavioral patterns (e.g., Maneja et al., 2013; Schmidt et al., 2017). In contrast, the latter involve examining species-specific "stereotypical" behavioral responses, usually following the application of a specific environmental stimulus such as introduction of an olfactory or visual cue. These approaches have more frequently identified apparent CO_2-induced alterations in behavior (e.g., Munday et al., 2010; Hamilton et al., 2014; Williams et al., 2019; but see Clark et al., 2020). These effects are hypothesized to be due to a physiological response to environmental hypercapnia in which alterations of bicarbonate ion concentrations in the blood and other extra-cellular fluids, consequently altering nerve signal transmission (Nilsson et al., 2012; Tresguerres and Hamilton, 2017).

Our observations, and those of previous studies of OA impacts on the behavior of gadid larvae are consistent with those broader patterns in the literature. In this study, we described the basic swim characteristics of larval pollock exhibiting "routine" activity in the experimental culture tanks. While these descriptions are not as detailed as those made by Maneja et al. (2013) in specialized observation arenas with Atlantic cod larvae, both studies found only minor effects of CO_2 levels on swim traits concluding that routine behavior was generally robust to the effects of CO_2. In contrast, applying the "response to stimulus" approach, Hurst et al. (2019) found significant changes in the behavior of pre-flexion larval Pacific cod in a horizontal light gradient. Unfortunately, pre-flexion walleye pollock larvae are very sensitive to handing, and attempts to perform the light gradient trials resulted in high mortality shortly after transfer (Hurst, unpublished data), so we were unable to provide a direct comparison to the Pacific cod observations. Such a test would have further clarified the roles of species-specific responses and experimental approaches in overall characterization of behavioral sensitivity to OA.

Lipid responses to acidification

The impact of CO_2 on larval lipids was subtle with no significant influence on total lipids or total lipids per DWT. However, we did observe an ontogenetic-dependent effect of CO_2 on larval walleye pollock lipid classes. At both weeks 2 and 4 we saw a significant elevation in sterols of walleye pollock at elevated CO_2 conditions. Cholesterol is the major sterol in larval fish and it is important, along with phospholipids, for maintaining the structural integrity of cell membranes (Parrish 2013). However, sterols also play an important role as precursors for corticosteroids that are up-regulated during the stress response in fish (Xu et al., 2018; Jones et al., 2020). Eggs and newly hatched larvae of Atlantic haddock (Melanogrammus aeglefinus) showed upregulation of genes for cholesterol synthesis following exposure to crude oil (Sørhus et al., 2020). The up-regulation of sterol metabolic pathways in larval fish following

469 exposure to high CO_2 conditions has not yet been documented, but is a conceivable response to metabolic
470 and osmoregulatory stress.
471 Triacylglycerols are the major storage lipid class in marine fish and storage of TAG can be used
472 as a sensitive indicator of nutritional status in marine larval fish because it is the first lipid class utilized
473 during periods of environmental stress or reduced food availability (Fraser 1989, Copeman & Laurel
474 2010). We found no evidence of upregulation of TAG synthesis in response to elevated CO_2 but rather
475 higher storage of TAG from dietary sources in larvae reared under ambient conditions (discussed below).
476 Our results for walleye pollock larvae exposed to 1230 µatm CO_2 are in contrast to previous studies on
477 Pacific cod (Hurst et al., 2019) and Atlantic cod (Frommel et al., 2012) where larvae were cultured under
478 higher CO_2 conditions (~1700 to 4,200 µatm, respectively). Neutral lipid storage in larval Atlantic cod
479 and the observations of TAG-filled lipid vacuoles only significantly increased in larvae from the highest
480 CO_2 treatment compared to the control (Frommel et al., 2012). The Atlantic cod larvae from that
481 experiment have since been shown to have had upregulation of genes associated with fatty acid synthesis
482 and glycogen metabolism (Frommel et al., 2020), elucidating a mechanism for the accumulation of
483 neutral storage lipids. It is likely that the level of lipid dysregulation is dependent not only on the
484 ontogenetic stage but also the level of CO_2 exposure pointing to the importance of careful considerations
485 of experimental design in evaluating the severity of sub-lethal effects in larval fishes.
486 The expression of TAG content relative to sterols (STs), a structural lipid class serving as a scale
487 for overall body size, has previously been used as a condition index for marine fish and bivalve larvae
488 (Fraser 1989, Suthers 1998). TAG/ST ratios in our study did not increase with ontogeny indicating that
489 larvae were prioritizing rapid growth over energy storage. The significant interactive effect of CO_2 on the
490 TAG/ST ratio in larvae was driven by combined effects of reduced storage lipids in high CO_2 larvae at
491 week 4 with the overall higher levels of ST in high CO_2 larvae. Given increasing evidence that crude oil,
492 high CO_2 or other environmental stressors may dysregulate normal lipid synthesis pathways for both TAG
493 and ST (Frommel et al., 2012, Frommel et al., 2014, Sørhus et al., 2017, Laurel et al., 2019, Frommel et
494 al., 2020), this lipid-based condition index should be interpreted with caution for assessment of impacted
495 larval survival potential.
496 As with total lipids and lipid classes, there are varied accounts related to the effect of elevated
497 CO_2 on larval FA density and proportions (Frommel et al., 2012; Díaz-Gil et al., 2015, Murray et al.,
498 2017). These varied accounts are likely due to the specifics of each study, such as diet and ontogenetic
499 stage examined as well as species-specific variation in CO_2 sensitivity. Fatty acids are important
500 components of all acyl lipid classes which include TAG and PL as well as other minor classes such as
501 FFA and mono- and diacyl-glycerols (Parrish 1988). Analyses of the FA proportions from a total lipid
502 pool should therefore be advanced with thoughtfulness because this type of metric does not differentiate

changes that are due to dietary storage versus those due to membrane or eicosanoidal-type effects (discussed in Copeman et al., 2018, Hurst et al., 2019). The molecular fatty acid species composition of neutral lipid storage in fish is generally representative of diet, while the FA composition of the phospholipids are genetically determined and species-specific (Bell & Dick 1991, MacPherson et al., 1998, Budge et al., 2006). Examination of the multivariate lipid response in walleye pollock larvae showed differentiation of larvae in the high CO_2 treatment from week 2 to week 4 mostly based on lipid classes (TAG to ST & PL) while differentiation of larvae in the ambient treatment from week 2 to week 4 reflected assimilation of dietary fatty acids from enriched rotifers. Larval fish are unable to synthesize significant quantities of long chain essential fatty acids such as 22:6n-3, 22:5n-6 and 20:5n-3 (Sargent et al., 1999, Copeman et al., 2002). At week 4, larvae from the ambient treatment had higher quantities of the polyunsaturated fatty acids (PUFA) that are found in higher concentrations in their Algamac-enriched rotifer prey (22:5n-6 and 18:2n-6) than in newly hatched Pacific cod larvae (Copeman & Laurel 2010, Laurel et al., 2010). In contrast, larvae from the high CO_2 treatment did not assimilate these dietary PUFA to the same degree indicating reduced feeding success, digestion, or lipid assimilation at week 4.

Frommel et al. (2020) has shown up-regulation of genes related to lipid metabolism in Atlantic cod larvae, but not juveniles, under extreme CO_2 conditions indicating an ontogenetic-dependent lipid response. Here we found evidence of increased sterols in high CO_2 larvae, but the genes involved in their synthesis have not yet been targeted for study in responses to high CO_2 conditions. We hypothesize that differences in fatty acid proportions in walleye pollock larvae observed here were a result of differential dietary lipid assimilation success. Further experimentation across a range of projected CO_2 exposure levels, targeted gene expression, and lipid-class specific fatty acid analyses is needed to more fully understand the impact of OA on differential synthesis and storage of specific fatty acids in marine fish larvae.

Conclusions

The results and discussions presented above illustrate the difficulties of integrating information on the range of potential effects of high CO_2 to develop broad, comprehensive understanding of species-specific sensitivity and the implications of ongoing OA to marine ecosystems and resource-dependent industries and communities. Given the range of potential physiological, behavioral, and ecological responses, research programs (even on well-studied groups such as marine gadids) must select a subset of potential responses and draw upon patterns observed in related species. Responses are frequently shown to be dependent upon developmental stage, complicating the desire for a universal evaluation for a given species. In addition, these responses can appear contradictory or counter-intuitive. For example, while we observed lower rates of swim bladder inflation in walleye pollock larvae exposed to high CO_2, we did not

observe lower survival rates which might be expected among such fish. It is unknown if the lower rates of swim bladder inflation would have had carry-over effects on growth or survival in later larval stages. Moreover, the benign conditions of the laboratory culture environment may have masked the potential impacts of this morphological deficit on foraging success or predator avoidance that would be expressed among wild fish. Finally, the relationship between responses in multi-trait studies is often not clear. In walleye pollock and Pacific cod larvae it is unclear whether the observed behavioral responses act independently of, or are a contributing factor to observed growth, energetic, and survival differences. We suggest that future studies continue to examine multiple traits to independently evaluate the generality of sensitivity and evaluate the potential for sub-lethal and carry-over effects of CO_2 exposure during the early life stages.

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Author contributions TPH, LAC, and MLK designed the experiment. All authors collected the data. TPH, LAC, JFA and MS analyzed the data. TPH led manuscript preparation with contributions from LAC, JFA, and MLK. All authors reviewed, edited, and approved the manuscript for submission.

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Compliance with ethical standards

Conflicts of interest The authors have no conflicts of interest.

Ethical approval This research was carried out in accordance with all applicable institutional and national guidelines at the time that the study was conducted. Ethical review and approval was not required for the animal study because this experiment was conducted in NOAA's Alaska Fisheries Science Center

Laboratory in Newport, Oregon. NOAA National Marine Fisheries Service does not have an Institutional Animal Care and Use Committee (IACUC) approval processes for research on fishes. All work followed American Fisheries Society policies on the Guidelines for Use of Fishes in Research (https://fisheries.org/docs/policy_useoffishes.pdf) and AVMA (American Veterinary Medical Association) Guidelines on Euthanasia (https://olaw.nih.gov/sites/default/files/Euthanasia2007.pdf).

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828

829 **Table 1.** Conditions during experimental exposures of walleye pollock (*Gadus chalcogrammus*) eggs and
830 larvae to projected ocean acidification. Temperature was measured daily in each fish tank; carbonate
831 system parameters (dissolved inorganic carbon, DIC; total alkalinity, TA) were measured from preserved
832 bottle samples taken three times per week from conditioned seawater supply and used to calculate pH and
833 pCO_2. Values shown are means ± 1 SD.

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Experiment-Treatment	Temp. (°C)	Salinity	DIC (µmol kg^{-1})	TA (µmol kg^{-1})	pH (total pH scale)	pCO_2 (µatm)
Ambient CO_2 treatment						
Egg incubation	5.9 ± 0.4	33.1 ± 1.1	2127.1 ± 68.3	2265.5 ± 63.5	8.01 ± 0.05	430 ± 70
Larval phase	6.3 ± 0.5	31.2 ± 0.7	2000.4 ± 35.4	2120.7 ± 43.1	8.01 ± 0.06	423 ± 126
Combined phases	6.3 ± 0.5	31.5 ± 1.0	2022.7 ± 72.6	2146.2 ± 72.6	8.01 ± 0.06	424 ± 117
High CO_2 treatment						
Egg incubation	5.9 ± 0.4	33.7 ± 0.2	2305.7 ± 37.5	2315.8 ± 22.3	7.60 ± 0.07	1211 ± 183
Larval phase	6.4 ± 0.5	31.2 ± 0.7	2141.3 ± 44.0	2133.3 ± 41.0	7.57 ± 0.07	1232 ± 225
Combined phases	6.3 ± 0.5	31.5 ± 1.1	2162.5 ± 70.4	2156.9 ± 73.3	7.57 ± 0.07	1228 ± 217

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838 Table 2. Body size and condition metrics of larval walleye pollock (*Gadus chalcogrammus*) reared at
839 ambient and high CO_2 levels. Values are the mean (SD) of four tank mean values within each treatment.
840 Asterisks indicate a significant difference between ambient and high CO_2 treatments ($p < 0.05$) based on
841 linear mixed models including replicate tank as a random factor.
842
843

Age	CO_2 treatment	SL (mm)	BD (mm)	K_{DB}	ED (mm)	DW (mg)	K_{DW}
2 weeks	Ambient	5.98	0.35	-0.25	0.40	0.12*	-1.30*
		(0.18)	(0.018)	(0.84)	(0.009)	(0.014)	(0.60)
	High	5.95	0.35	0.51	0.41	0.14	0.33
		(0.22)	(0.015)	(0.78)	(0.012)	(0.013)	(0.90)
4 weeks	Ambient	7.12	0.46	0.77	0.49	0.25	0.10
		(0.14)	(0.017)	(0.92)	(0.011)	(0.023)	(1.70)
	High	7.29	0.47	-0.25	0.50	0.26	-0.97
		(0.16)	(0.011)	(0.95)	(0.008)	(0.026)	(0.85)

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Table 3: Total lipid, lipid classes, and fatty acid composition of walleye pollock larvae (*Gadus chalcogrammus*) after 2 weeks and 4 weeks of experimental rearing under ambient and high CO_2 conditions as well as the fatty acid composition of enriched rotifers. Data are mean ± SD.

Age	Enriched Rotifers	2 weeks	2 weeks	4 weeks	4 weeks
CO_2 treatment		Ambient	High CO_2	Ambient	High CO_2
n of tanks	3	4	4	4	4
DW per larvae (µg)		121.6 ± 13.72	138.3 ± 13.0	247.8 ± 22.6	258.2 ± 25.4
Total lipids per larvae (µg)		10.3 ± 1.06	11.7 ± 1.0	19.2 ± 1.2	19.6 ± 2.8
Total lipids per DW (µg·mg^{-1})		85.1 ± 4.0	84.7 ± 9.6	77.9 ± 5.5	77.3 ± 16.9
% Triacylglycerols		7.3 ± 0.9	8.8 ± 0.9	7.0 ± 0.8	5.2 ± 0.3*
% Free fatty acids		4.2 ± 1.0	3.7 ± 0.9	3.1 ± 0.8	2.6 ± 0.6
% Sterols		11.7 ± 0.3	11.7 ± 0.2	12.5 ± 0.4	13.8 ± 1.2
% Polar lipids		76.9 ± 1.7	75.8 ± 1.5	77.4 ± 0.7	78.4 ± 0.9
TAG/ST		0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Total fatty acids per DW (µg·mg^{-1})		41.5 ± 3.1	42.2 ± 3.9	43.0 ± 3.3	31.6 ± 10.4
Total fatty acids per individual (µg)		5.0 ±0.4	5.3 ± 0.6	10.6 ± 0.5	8.0 ± 2.1*
%14:0	4.3 ± 0.2	2.0 ± 0.1	2.0 ± 0.0	1.9 ± 0.1	1.9 ± 0.2
%16:0	17.3 ±0.6	20.8 ± 0.3	19.9 ± 0.5	18.8 ± 0.4	21.1 ± 0.5
%18:0	1.8 ± 0.0	7.6 ± 0.1	7.2 ± 0.3	7.2 ± 0.2	8.0 ± 0.6
∑SFA	24.7 ± 0.9	31.8 ± 0.4	30.1 ± 0.9	29.1 ± 0.5	32.6 ± 0.9
%16:1n-7	3.1 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.7 ± 0.1	1.8 ± 0.0
%18:1 n-9	4.6 ± 0.0	7.9 ± 0.1	7.3 ± 0.3	6.6 ± 0.1	7.3 ± 0.4
%18:1 n-7	1.0 ± 0.0	3.0 ± 0.1	2.9 ± 0.2	2.5 ± 0.1	2.8 ± 0.2
%20:1 n-9	1.2 ± 0.0	2.0 ± 0.1	1.7 ± 0.1	1.2 ± 0.0	1.5 ± 0.2
∑MUFA	12.9 ± 0.1	18.2 ± 0.4	17.4 ± 0.4	14.7 ± 0.4	16.2 ± 0.4
%18:2n-6	6.3 ± 0.1	4.0 ± 0.1	4.0 ± 0.1	4.4 ± 0.1	4.2 ± 0.3
%20:4n-6	2.8 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	4.4 ± 0.1	4.3 ± 0.1
%20:5n-3	8.7 ± 0.1	5.5 ± 0.1	5.7 ± 0.2	5.1 ± 0.2	4.8 ± 0.1
%22:5n-6	7.1 ± 0.2	3.9 ± 0.2	4.3 ± 0.3	5.3 ± 0.2	4.7 ± 0.3

%22:5n-3	4.3 ± 0.1	2.3 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.2 ± 0.1
%22:6n-3	24.3 ± 0.6	24.8 ± 0.6	25.7 ± 0.5	26.5 ± 0.6	24.6 ± 1.0
∑PUFA	60.9 ± 1.0	50.0 ± 0.9	51.6 ± 1.2	55.1 ± 0.9	50.1 ± 0.9

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853 **Figure captions**

854

855 **Fig. 1** Swim bladder inflation in walleye pollock larvae. A, B) Histology (H&E), sagittal sections. A =
856 fish with inflated swim bladder (SB). P = pancreas, N = notochord. B = uninflated swim bladder with
857 prominent epithelium (arrow). Bar = 100 µm. C-E) whole mounts. C with inflated swim bladder (SB). D
858 with uninflated swim bladder. E = eye, L = liver, In = intestine, B = notochord. Bar = 1 mm

859

860 **Fig. 2** Survival patterns of walleye pollock embryos and larvae at ambient and elevated CO_2 levels. Fates
861 were based on estimated initial egg densities, daily counts of egg mortalities, and count of larvae
862 surviving until the end of the experiment

863

864 **Fig. 3** Swim patterns of walleye pollock larvae as a function of age and CO_2 level. Panels show the
865 number of turning movements in relationship to the number of forward swimming movements. Points
866 represent the movement patterns of randomly selected fish in the culture tanks observed for 1 minute

867

868 **Fig. 4** Swim bladder inflation rates (top) and swim bladder size (bottom) at ambient and elevated CO_2
869 levels. At 4 weeks of age, 25 fish were sampled from each culture tank and examined for the presence and
870 size of the swim bladder. In the top panel, lines represent logistic regression (± s.e.) of successful swim
871 bladder inflation on body size

872

873 **Fig. 5** Lipid levels of larval walleye pollock at 2 and 4 weeks at ambient and elevated CO_2 levels. Values
874 are mean (± SE) of four replicate rearing tanks in each treatment. Tukey's pairwise test was used for post-
875 hoc comparisons on interactive effects of week*CO_2

876

877 **Fig. 6** Patterns of variation in lipid classes and fatty acid composition in 2- and 4-week old walleye
878 pollock larvae reared at ambient and high CO_2 levels. Larval samples are displayed using nMDS (non-
879 metric multidimensional scaling) on Bray-Curtis similarities of square-root transformed fatty acid and
880 lipid class proportion data. Variables included in the analysis were major lipid classes triacylglycerols
881 (%TAG), free fatty acids (%FFA), sterols (%ST), and phospholipids (%PL), individual major fatty acids
882 (>2% of the total), and the sums of the saturated (SFA), monounsaturated (MUFA) and polyunsaturated
883 (PUFA) fatty acids

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886 Figure 1.

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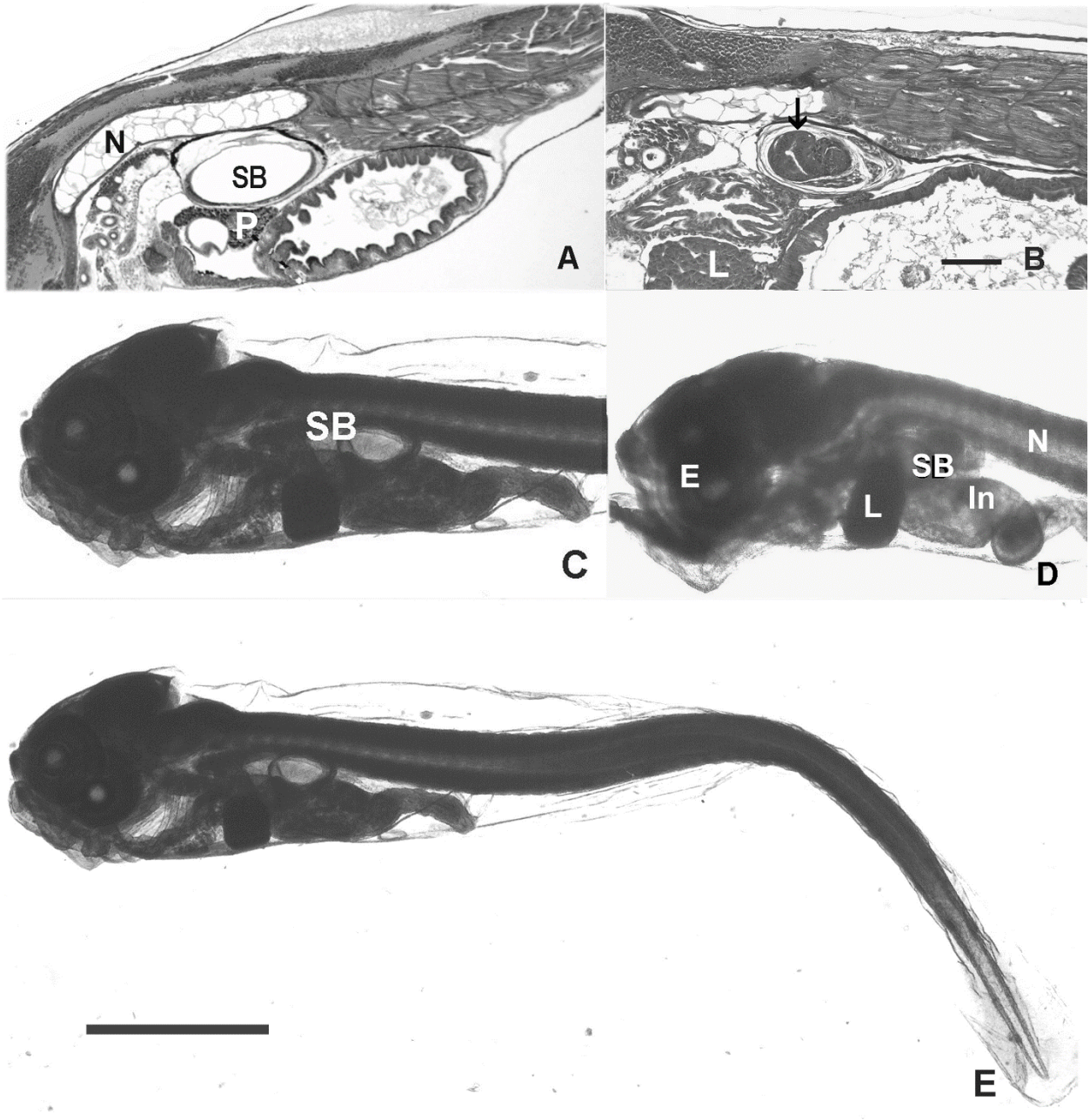

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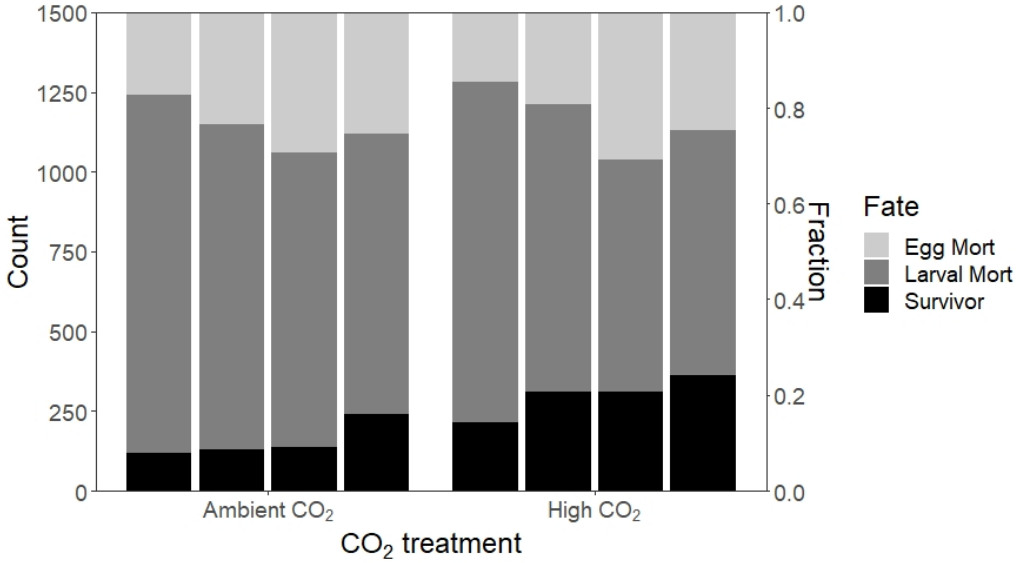
Figure 2.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Survivor
Ambient CO2
High CO2
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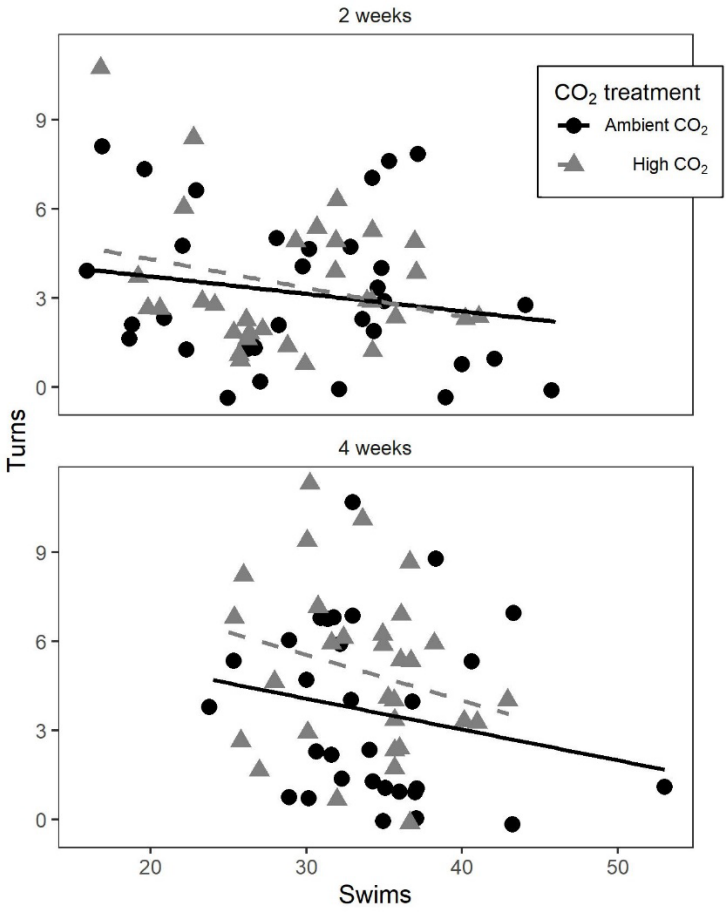
Figure 3.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CO_2 treatment
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910 Figure 4.

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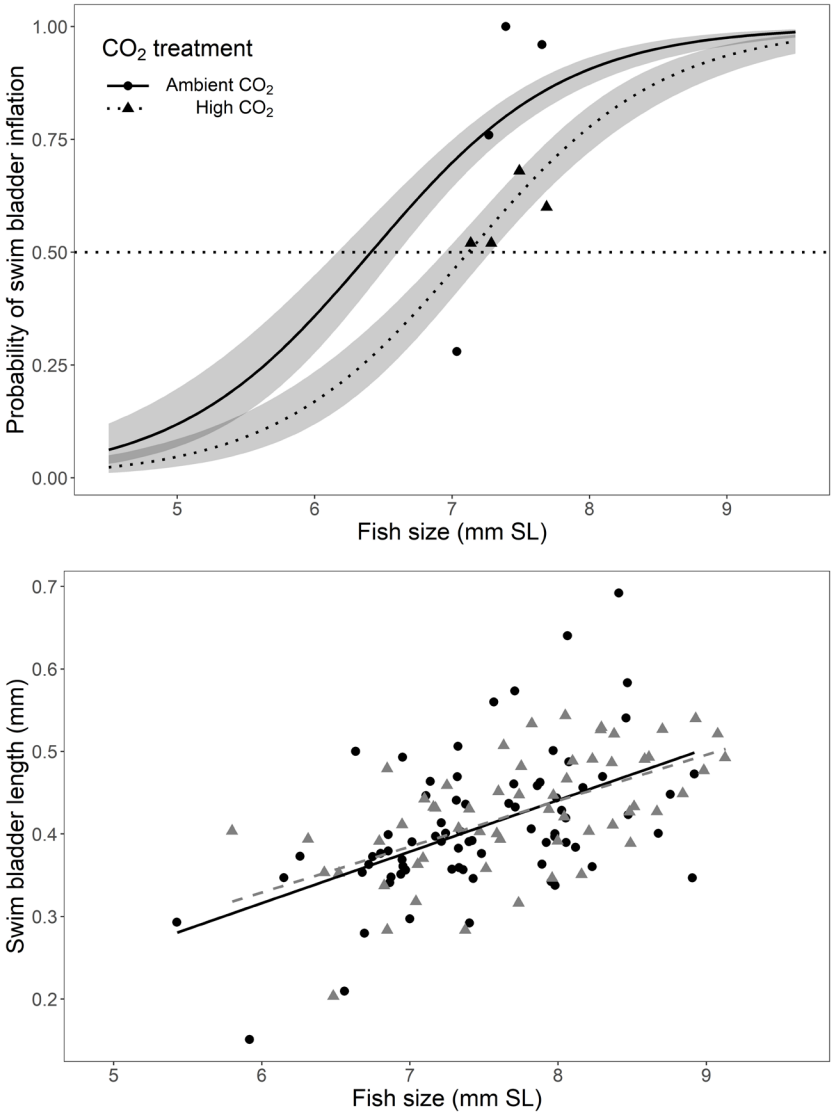

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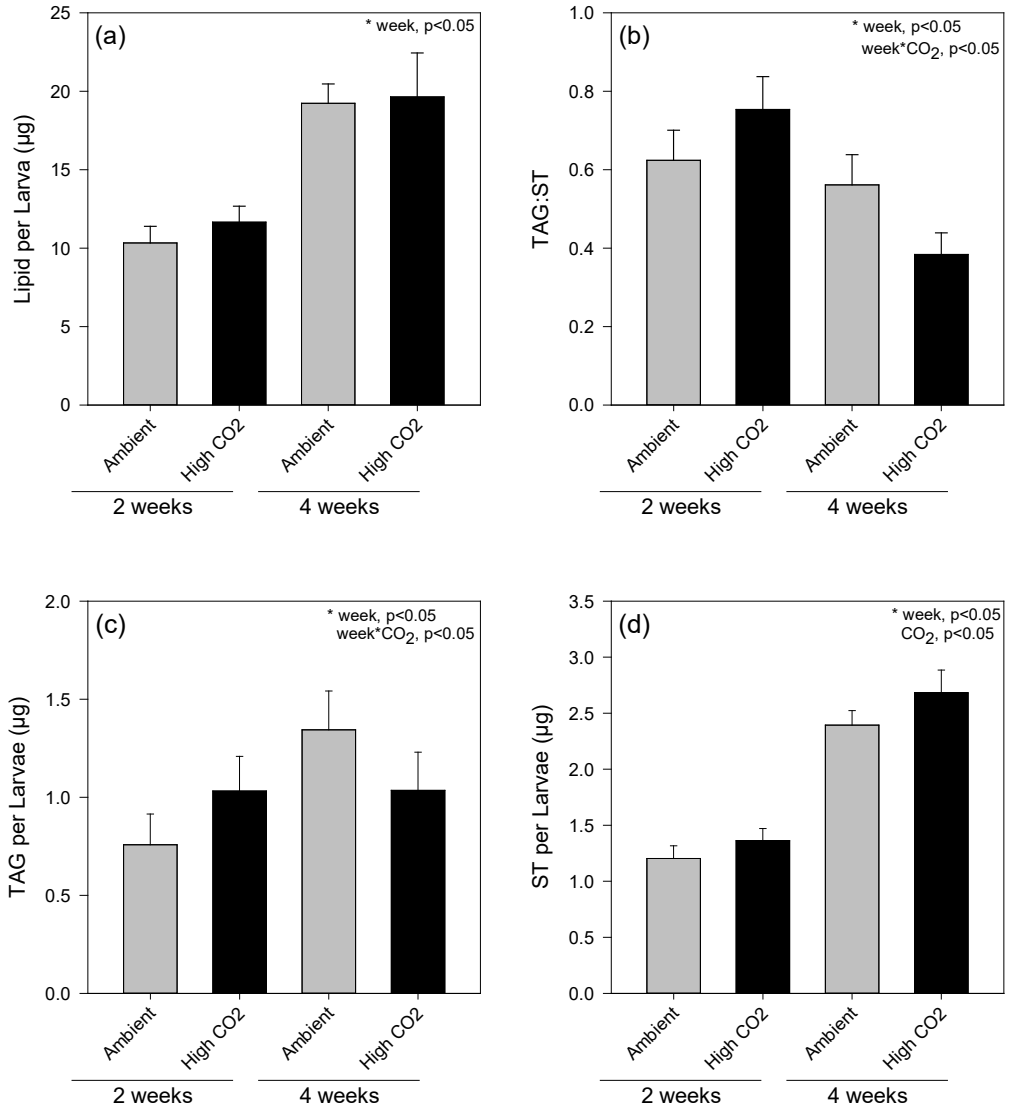
916 Figure 5.



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919 Figure 6.

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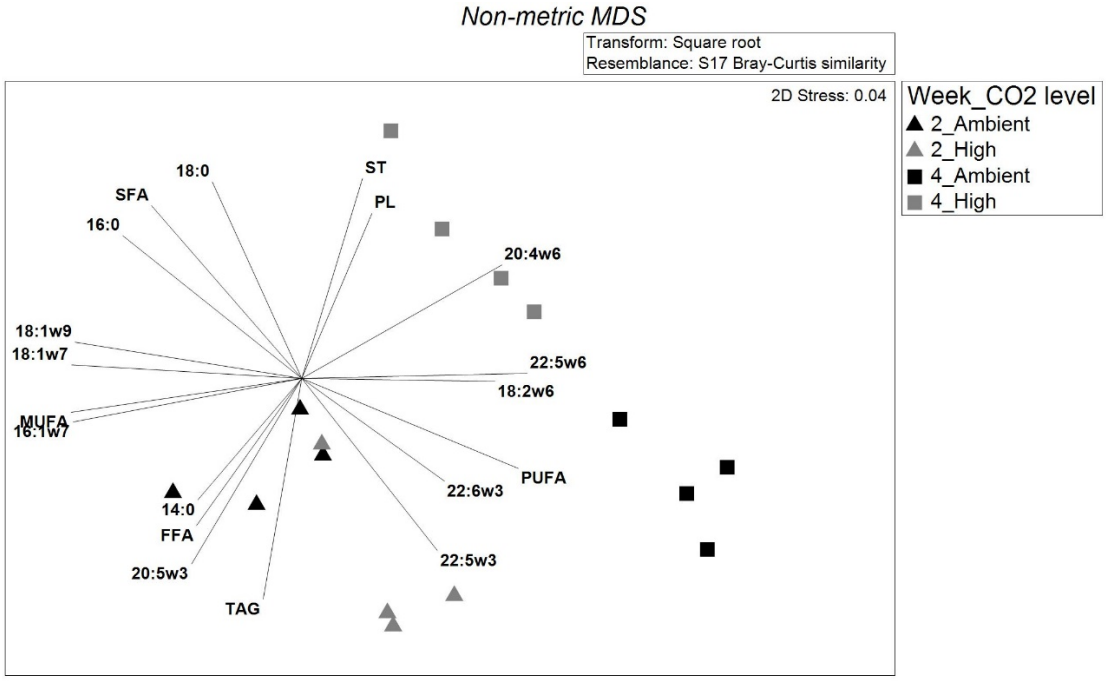

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