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Elevated CO_2 does not exacerbate nutritional stress in larvae of a Pacific flatfish

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Running head: CO_2 and prey abundance effects on flatfish larvae

ABSTRACT

Multiple aspects of climate change are expected to co-occur such that ocean acidification will take place in conjunction with warming and a range of trophic changes. Previous studies have demonstrated that nutritional condition plays a significant role in the responses of invertebrates

to ocean acidification, but similar studies have yet to be conducted with marine fishes. In this study, we examined the potential interactive effects of elevated CO_2 levels and nutritional stress on the growth and development of northern rock sole (*Lepidopsetta polyxystra*). Separate experiments examined the effects of these two environmental stressors during the pre-flexion (3-31 days) and post-flexion (31-87 days) larval stages. In both stages, larval feeding regime has a much larger impact on growth rates than did CO_2 level, and there was no observed interaction between stressors. By 31 days post-hatch, larvae in the high feeding treatment were 84.2% heavier than the fish in the low feeding treatments, but there was no significant effect of CO_2 level on body size or condition. While overall growth rates were faster during the pre-flexion stage, the effects of food limitation were greater for post-flexion larvae undergoing metamorphosis, with high feeding treatment fish being 3.3 times as heavy as fish in the low feeding treatments. These results have important implications for understanding the impacts of the multi-faceted nature of climate change on population productivity of commercial fish species in the North Pacific.

Key words: climate change, feeding environment, flatfish, growth, metamorphosis, ocean acidification

INTRODUCTION

Understanding the impacts of environmental variation on the population productivity of resource species is a fundamental issue in contemporary fisheries science. It is important to understand how naturally-varying environmental factors interact with long-term anthropogenic impacts of climate conditions at regional and global scales. The (comparatively) recent recognition that anthropogenic increases in CO_2 levels are not restricted to the atmosphere have prompted concern that ocean acidification may be an additional stressor disrupting fishery production and ecosystem dynamics (Denman et al., 2011; Haigh et al., 2015). Given the importance of early life growth and survival rates on population dynamics of marine fish and invertebrates, recent research effort has focused on evaluating the impacts of elevated CO_2 levels on the egg, larval, and juvenile stages for a diversity of marine species.

Experiments with marine fishes have found varying effects of ocean acidification. Most experiments with juveniles have found them to be robust to the energetic effects of hypercapnia

(Ishimatsu et al., 2008; Hurst et al., 2012). While, some studies with fish larvae have observed negative impacts on growth and survival (Baumann et al., 2012; Pimentel et al., 2014) or increased incidences of developmental anomalies (Chambers et al., 2014; Frommel et al., 2014), other studies have found no such evidence of negative effects of elevated CO_2 levels on larvae (Munday et al., 2011; Bignami et al., 2014). In studies of Alaskan fishes, laboratory experiments at elevated CO_2 levels indicated a trend toward higher mortality rates and reduced condition levels in post-flexion larvae of northern rock sole (Hurst et al., 2016), but not walleye pollock (Hurst et al., 2013).

It is well recognized that changes in the prevailing climate will lead to simultaneous changes in multiple aspects of the environment, such that acidification will occur along with warming temperatures as well as changes in stratification, deoxygenation, and precipitation patterns. Consequently, studies have started to examine the potential for interaction among factors, pairing elevated CO_2 with other environmental stressors in multi-factor experiments. To date, most of these factorial experiments have paired high CO_2 with elevated temperatures (e.g., Munday et al., 2009; Ko et al., 2015). However, throughout much of their range, most species are not living near their thermal tolerance limits and may not face a physiological risk from increased temperatures. In these cases, climate effects other than temperature may exert the strongest influences on population dynamics (Rijnsdorp et al., 2009). Because they have limited energetic reserves, the survival of marine fish larvae is especially sensitive to the foraging environment encountered during the first few weeks of life. The idea that interannual variation in the spatial pattern and seasonal timing of prey production can be a significant determinant of year-class success is encapsulated in both the “Critical Period Hypothesis” (Hjort, 1914) and the “Match-Mismatch Hypothesis” (Cushing, 1975). A mismatch occurs when prey availabity is lower than necessary to support the growing cohort of larvae or when the phenology of fish reproduction occurs such that prey are not available when larvae complete absorbtion of their yolk resreves and must start exogenous feeding. If elevated CO_2 imposes additional energetic stress on larval fish or disrupts patterns of prey production, ocean acidification could exacerbate the risk of “prey mismatches” during critical periods when fisheries recruitment is determined.

Several studies on corals and other invertebrates have demonstrated that the effects of ocean acidification on growth, survival, and other traits are sensitive to the nutritional status or foraging condition of the animals. Mortality of juvenile orange cup corals (*Balanophyllia elegans*) increased at elevated CO_2, but this effect was partially mitigated by high feeding rates

(Crook et al., 2013). Similarly, Atlantic golf ball coral (*Favia fragum*) and staghorn coral (*Acropora cervicornis*) were able to maintain growth rates under elevated CO_2 levels while feeding exogenously, but not when relying exclusively on photosynthetic symbionts for nutrition (Drenkard et al., 2013; Towle et al., 2015). In both oysters (*Ostrea lurida*) and mussels (*Mytilis edulus*) the negative effects of elevated CO_2 levels on growth were smaller than the effects of prey level manipulation (Hettinger et al., 2013; Thomsen et al., 2013). These results suggest a potentially important role of nutritional condition and feeding status on the responses of marine organisms to elevated CO_2 levels associated with ocean acidification. However, to date there have been no published studies examining the interactive effects of CO_2 level and nutritional in a marine fish.

A commercially important flatfish in the North Pacific Ocean, northern rock sole (*Lepidopsetta polyxystra*) spawn semi-adhesive, demersal eggs; pelagic larvae occur in the upper 30 m of the water column (Lanksbury et al., 2007). Following metamorphosis, fish settle into shallow coastal habitats at sizes of 12 – 18 mm TL (Laurel et al., 2015). Previous work (Hurst et al., 2016) found that elevated CO_2 levels had modest effects on the survival, growth, and condition of larval northern rock sole. However, these experiments were conducted under food-replete conditions and it is unknown if nutritional stress would exacerbate the effects of elevated CO_2.

In this study we present two experiments examining the influence of prey availability and elevated CO_2 levels on the growth, development and survival of larval northern rock sole. Experiments were conducted on both pre-flexion (Experiment 1, 28 d exposure) and post-flexion (Experiment 2, 56 d exposure) larvae to evaluate the potential for stage-specific interactive effects of ocean acidification and nutritional stress in this representative flatfish species.

METHODS

Experimental System

Larvae were reared in a flow-through system under controlled temperature and pH conditions (modified from Hurst et al., 2012). Ambient temperature and chilled seawater were mixed to achieve 8°C in two conditioning tanks. Injection of CO_2 into one of these conditioning tanks was regulated with a pH probe (Honeywell Durafet III) to achieve the high CO_2 treatment (pH ~7.6). The second tank was maintained at the ambient CO_2 level (pH ~ 8.0). The target pH of 7.6 corresponds to a CO_2 level of approximately 1100 µatm, chosen to reflect expected

conditions in the North Pacific Ocean and Bering Sea in the next 100 years (Mathis et al., 2015). Water from these conditioning tanks was pumped to two header tanks each for the "high" and "ambient" CO_2 treatments. Each header tank gravity-fed four 100-L rearing tanks at 500-700 mL/min (8 tanks in each pH treatment). An identical pH meter was used to measure pH in the high and low CO_2 treatments daily and to measure pH in all rearing tanks 3-4 times per week. In addition, carbonate conditions during experiments were more completely characterized by chemical analysis of water samples drawn from each treatment header tank 2-3 times per week. Water samples were poisoned with $HgCl_2$ and analyzed at the Ocean Acidification Research Center at the University of Alaska at Fairbanks for dissolved inorganic carbon (DIC) and total alkalinity (TA) using a VINDTA 3C (Versatile Instrument for the Determination of dissolved inorganic carbon and Total Alkalinity) coupled to a UIC 5014 coulometer. These data were used to calculate the pH, pCO_2, and carbonate mineral saturation states (Ω) of the rearing waters using the program developed by Lewis and Wallace (1998; Table 1).

Parental broodstock

Eggs for these experiments were produced by a captive broodstock of northern rock sole at the Alaska Fisheries Science Center's laboratory in Newport, Oregon. Fish for the broodstock were collected as adults (32-40 cm total length) from coastal waters of Kodiak Island, Alaska (57°46' N 152°21'W) and reared in the laboratory (see Laurel and Blood, 2011 for additional details). The fish were held in a 6-m tank under seasonally varying temperature and photoperiod. Temperatures in the spawner tank were maintained at 7-10°C during the summer and reduced to 4-5°C during the winter; light was provided on a seasonal cycle varying from 14.5 h light during the summer to a minimum of 9.5 h prior to the spawning season. During the spawning season, water samples were collected from the broodstock tank once per week and analyzed to determine CO_2 (415 ± 76 µatm SD) and pH (7.99 ± 0.08) conditions as described above. Although we did not monitor pH or CO_2 levels in the adult broodstock tanks regularly outside the spawning season, measurements of ambient seawater during experiments in Hurst et al. (2012) demonstrate strong seasonal variation. During the summer when coastal upwelling brings CO_2–rich water to the surface (Gruber et al., 2012) ambient CO_2 levels can reach more than 700 µatm during prolonged upwelling events.

Male and female fish were allowed to ripen naturally without the use of hormonal injections. When females showed external signs of egg ripening (distended abdomen), they were

captured from the tank and checked for spawning condition by gently squeezing the abdomen. If eggs did not flow freely, the fish was returned to the tank and re-checked at 3-4 day intervals. When eggs flowed freely, they were collected in a clean, dry container and fertilized with milt from three male fish randomly selected from the broodstock. Gametes were mixed for 1 minute and repeatedly rinsed with ambient pH-seawater to remove excess milt and disperse egg clumps. Eggs were incubated in a single layer in a 175 L flow-through tank at 6°C and ambient CO_2 levels (~ 350 µatm).

Experiment 1 – Pre-flexion (days 0 -29)

Three days after the estimated mid-point of the hatch cycle (operationally defined as 3 days post-hatch, DPH), yolk-sac larvae were gently scooped from the surface water of the egg incubation tank, warmed to 8°C over a 1-hour period and introduced to larval rearing tanks. Each 100 L rearing tank was stocked with approximately 2,500 larvae. These stocking densities are similar to those used by Hurst et al. (2016) and below levels expected to induce density-dependent effects on growth rates. Larvae were reared in black, 100-L tanks with weak upwelling circulation maintained by light aeration and positioning the in-flow (~500 $mL \cdot min^{-1}$) at the bottom center of the tank. Light was provided by overhead fluorescent bulbs on a 12:12 h light:dark photoperiod at a level of 6.7 $\mu E/m^2s$ at the water surface. Prey was introduced 1 day after stocking. Larvae were reared on rotifers (*Brachionus plicatilis*) enriched with Algamac 3050 (Aquafauna, Hawthorne, CA), initially supplied at densities of 5 $prey \cdot mL^{-1}$ twice daily.

One day after fish stocking, CO_2 injection was initiated, reducing pH levels in the high CO_2 treatment. On the second day after stocking, tanks were randomly assigned to three feeding treatments (low $n = 3$; medium $n = 2$; high $n = 3$) within each CO_2 treatment. Feeding rates in the medium food treatment were maintained at 5 $prey \cdot mL^{-1}$, provided twice daily (similar to feeding rates used in Hurst et al., 2016); the low food treatment was reduced to 0.5 $prey \cdot mL^{-1}$, provided twice daily; and the high food treatment increased to 5 $prey \cdot mL^{-1}$, provided four times daily.

Over the next 4 weeks, 15 fish were sampled from each rearing tank for measurements at weekly intervals. After 4 weeks, all remaining fish in each replicate were counted. Fish were then pooled across the replicate tanks within each CO_2 and feeding treatment (low and high food only) and 300 of these fish were restocked in a single tank for extended rearing. No fish were transferred across treatments for the extended rearing phase. Fish in this extended rearing phase were sampled for measurements at 48, 62, 76, and 90 DPH. Sampled larvae were anaesthetized

with MS-222 and digitally photographed under a dissecting microscope and measurements were made of standard length (SL) and myotome height at the anus (MH). The fractional deviation from the overall relationship between MH and SL was calculated for each fish individually and used as in index of larval condition (MH_{dev}). Stage of developmental flexion was determined for each fish according to the criteria of Hawkyard et al. (2014): Stage 1) no signs of flexion; Stage 2) formation of a "node" near the posterior end of the notochord, but no bending of the notochord; Stage 3) bending of the notochord and extension of the notochord into the caudle peduncle; Stage 4) bent of the notochord not extending past the caudle peduncle. After photographing, subsampled larvae were pooled into groups of 5 larvae (n = 3/replicate) and dried for 24 hours at 50°C for determination of dry weight (DW). Because DW was not measured separately for each fish, dry weight condition factors (DW_{dev}) were estimated for each replicate tank on each sampling date as the percent deviation from the observed relationship between mean SL and mean DW. The underlying relationship was described by fitting a negative exponential smoothing function to the SL-DW data as ontogenetic patterns in growth and development of the larvae resulted in systematic departures from simple exponential, power, or polynomial functions (based on patterns in residuals). Mean size of fish in each replicate tank was used as the level of observation in statistical analyses.

Fish size (SL, MH, and DW) and condition (MH_{dev} and DW_{dev}) were analyzed with 3-way ANOVA with feeding treatment, CO_2 level, and age as main effects. All 2-way interactions were included in the model. In addition, growth rates in length (G_L, mm/d) and mass (weight-specific growth G_M, /d) were calculated from linear regression of mean length and ln-transformed mass against fish age for each rearing tank. Growth rates were compared across treatments with 2-way ANOVA.

Experiment 2 – Post-flexion larvae

Northern rock sole eggs were incubated and allowed to hatch as described above. After hatching, yolk-sac larvae were transferred from the egg-incubation tanks to larval rearing tanks (n=8). Fish were reared at 6-7°C for 4 weeks under ambient CO_2 levels. Initial rearing at ambient CO_2 levels was done to isolate the stage-specific effects of elevated CO_2 level on late-stage NRS larvae (see Hurst et al. 2016, for experiment rearing NRS from hatch to settlement at elevated CO_2). Fish were fed enriched rotifers at 5 prey·mL^{-1} twice daily. Microparticulate dry food (Otohime A, Reed Mariculture) was provided twice daily starting at 21 DPH. After 29 days, fish

were captured from these rearing tanks and randomly distributed across 12 identical tanks for subsequent rearing under ambient and elevated CO_2 conditions. Fish were stocked at densities of 300 fish per tank. Within each CO_2 treatment, 3 tanks were randomly assigned to either a low or high food treatment, with the change in feeding rates initiated 1 day after transfer. Feeding rate in the low food treatment was reduced to 0.5 prey·mL^{-1} provided twice daily and microparticulate dry food 1 time per day. Feeding in the high food treatment was increased to 5 prey·mL^{-1} provided four times daily and supplemented with microparticulate dry food 3 times per day.

Fish were sampled from each experimental tank after 12, 26, 40, and 54 days of exposure to experimental CO_2 and food conditions. After 54 days of rearing (at 84 DPH), the experiment was terminated and the number of fish remaining in each tank was determined. At each sampling, 10-12 fish were sampled from each tank and measured as described above. On the last date of sampling, all fish in the high food treatments had completed flexion (stage 4) and some had settled from the water column to the tank bottom. To avoid any possibility of bias associated with size differences between pelagic and demersal fish, the number of fish in each position was independently determined and both groups were sampled independently. Individual dry weights were determined independently for 5 fish from each sample. Condition metrics of MH_{dev} and DW_{dev} were calculated as fractional deviations from the underlying SL-MH and SL-DW relationships, respectively.

Mean size of fish in each replicate tank was used as the level of observation in statistical analyses. On the last sampling date, the mean size of fish in the high feeding treatment was based on measures of fish from both the water column and on the tank bottom, weighted by the fraction of fish in each group within each replicate tank. Fish size (SL, MH, and DW) and condition (MH_{dev} and DW_{dev}) were analyzed with 3-way ANOVA with feeding treatment, CO_2 level, and age as main effects. All 2-way interactions were included in the model. Growth rates were calculated for each tank and compared across treatments as described above.

RESULTS

Experiment 1 – Post-hatch larvae

During the first month of life, feeding level had a much larger effect on growth of pre-flexion northern rock sole than did CO_2 concentration (Fig. 1). This is reflected in the significant main effects of feeding treatment as well as the significant interaction with age on mean size expressed as SL, MH, and DW (Table 2). At 31 DPH, fish in the high feeding treatments were

1.38 mm longer and 84.2% heavier than the fish in the low feeding treatment. Fish in the medium treatment grew only slightly slower than fish in the high feeding treatment, being only 0.21 mm shorter and 15.1% lighter than fish in the high feeding treatment at 33 DPH. Conversely, there was no significant main effect of CO_2 treatment or other interactions with age or feeding treatment on fish size (SL, MH, or DW). These differences are reflected in growth rates calculated across the experiment (Fig. 2). Feeding level significantly affected G_L and G_M (2-way ANOVA, $p < 0.01$), but we observed no significant effects of CO_2 level or an interaction with feeding treatment ($p > 0.5$).

Responses in condition of pre-flexion larvae mirrored the patterns observed in growth. There were significant age and feeding treatment main effects on MH_{dev} (condition factor expressed as deviation from the relationship between MH and SL) as well as a significant interaction between these factors. Significant effects on DW_{dev} were confined to age and the interaction of age and feeding treatment. As with growth, there were no significant main or interactive effects of CO_2 treatment on larval condition (MH_{dev} or DW_{dev}).

Feeding treatment also had a significant effect on fish development (Fig. 3a). At 31 DPH, >90% of fish in the medium and high feeding treatments had reached flexion stage 2, whereas over 50% of fish in the low feeding treatments were still in flexion stage 1.

Survival varied within and across treatments. The highest number of fish surviving to 31 DPH occurred in the ambient CO_2-medium feeding treatment and the fewest in the high CO_2-high feeding treatment (Fig. 3). There were notably more survivors in the ambient CO_2 treatment at the two higher feeding levels than comparable feeding treatments in the high CO_2 treatments. However, due to the high variation in survival observed within treatments, there was no statistically significant effect of feeding treatment, CO_2 level, or their interaction on the number of fish in each tank after 28 days of exposure to experimental conditions (2-way ANOVA of log-transformed counts, $P > 0.10$).

After sampling on day 28 of the experiment (31 DPH), medium feeding treatments were discontinued. Surviving fish from the four other treatments were pooled across replicates and 300 fish stocked into one tank for each treatment for extended rearing under the same CO_2 and feeding conditions. Because there was no tank-level replication during this extended rearing, formal statistics were not performed. However, the earlier observed responses to CO_2 and feeding treatments persisted over this extended rearing period. At 87 DPH, fish in the low food treatments were 2.5 mm shorter and less than 1/3 the mass of the fish in the high food treatments.

In addition, there were more surviving fish in the high feeding treatments of each CO_2 level. Whereas approximately 50% of fish in the low feeding treatment had completed flexion (stage 4), all fish in the high feeding treatments, regardless of CO_2 level, had completed flexion (Fig. 3b).

There was only a minor effect of CO_2 treatment; fish in the high CO_2 treatment were 12% and 5% heavier than fish in the respective ambient CO_2 low and high feeding treatments. There was no apparent effect of CO_2 level on flexion stage. However, within the high feeding treatments, more fish in the ambient CO_2 treatment (46%) than in the high CO_2 treatment (33%) had completed flexion and settled to the tank bottom.

Experiment 2 - Post-flexion larvae (33 to 87 DPH)

In post-flexion larvae, the effects of feeding level had a much larger impact on growth and development than did CO_2 level (Fig 4). There was a significant main effect of feeding treatment on SL, MH, and DW as well as significant interactions with sampling date reflecting the increasing difference in size among feeding treatments (Table 3). Fish in the high feeding treatments averaged 1.77 mm longer and were 3.3 times as heavy as fish in the low food treatments after 54 days of rearing (87 DPH). These differences resulted in a significant feeding treatment effect on G_L and G_L ($p < 0.01$).

There was a small but significant main effect of CO_2 treatment on SL and MH among post-flexion fish (and a significant CO_2 treatment-feeding treatment interaction). High feeding treatment fish at high CO_2 were 0.29 mm longer than fish reared at ambient CO_2 levels, and low feeding treatment fish at high CO_2 were 0.07 mm longer than fish reared at ambient CO_2 levels. However, these size differences did not translate into a significant CO_2 effect on overall tank growth (G_L, $p = 0.36$). There was no significant main or interactive effect of CO_2 level on fish DW or G_W (Table 3).

Different responses were observed between the two measures of larval condition. There were significant effects of both CO_2 level and feeding treatment on MH_{dev}, as well as a significant interaction between age and feeding treatment. Interestingly, while both SL and MH were lower among fish in the low feeding treatments, MH_{dev} was higher in these fish than in fish in the high feeding treatments indicating a greater impact of prey availability on length than body depth. MH_{dev} was higher for fish reared at ambient CO_2 levels than those reared at elevated CO_2

levels. Conversely, the only significant effect on DW_{dev} was an interaction between age and feeding treatment.

Although differences were not statistically significant (ANOVA of log-transformed counts, $P > 0.10$), the high feeding treatments averaged 60% more survivors than the low food treatments (Fig 5). There was little difference between CO_2 treatments in survival or developmental stage. In the low food treatments, over 60% of fish were still in stage 3 of flexion, whereas all fish in the high food treatments had completed flexion (stage 4) and some had settled from the water column to the tank bottom. Again, there appeared to be little effect of CO_2 treatment on survival or stage of development within feeding treatment, although a higher percentage of fish among the high food tanks settled to the bottom under ambient than elevated CO_2 conditions (14.1% vs. 7.4%).

DISCUSSION

Population level productivity of marine fishes can be significantly influenced by abiotic factors as well as the foraging conditions that offspring encounter during their early life stages. Upon hatching, most fish larvae can only survive for a relatively short time on their endogenous yolk reserves, after which they must find sufficient suitable prey or succumb to starvation. Beyond that first-feeding stage, the foraging conditions encountered by larval fish significantly influence growth rate, regulating exposure to size-dependent predation mortality. The match-mismatch hypothesis suggests that rapid growth and high survival of a cohort of energetically-fragile fish larvae occurs when larval production is spatially and temporally coincident with their prey (Cushing, 1975; Durant et al., 2007). However, the physio-chemical environment can also influence the severity of these prey mismatches as larval fish are more vulnerable to mismatches of prey in warm water due to temperature-dependent increases in basal metabolism (Suneetha et al., 1999; Laurel et al., 2011). The potential role of elevated environmental CO_2 levels associated with ongoing ocean acidification has not been examined in this context.

In this study, we examined the relative impacts and potential interactions between prey availabity and CO_2 level, two aspects of the larval rearing environment that are predicted to shift under prevailing patterns of climate change. Concentrations of CO_2 in the surface waters of the world's oceans have risen demonstrably in association with increases in atmospheric CO_2 concentrations resulting in decreasing oceanic pH (Doney et al., 2009). Models uniformly predict a continuation of ongoing ocean acidification with the magnitude of predicted changes varying

with specific assumptions of the trajectory of anthropogenic CO_2 emissions (IPCC, 2014). In addition, regional oceanographic factors drive spatial variation in CO_2 and pH levels. In the Bering Sea and Gulf of Alaska (primary range of northern rock sole), acidification is expected to be exacerbated by sea-ice melt, increased river runoff, and organic matter respiration (Mathis, 2011; Mathis and Questel, 2013). The most recent estimates for these Alaska waters are that surface water pH has already decreased by 0.10 units and will decrease an additional 0.35 units by the year 2100 (see Mathis et al., 2015 for details).

The primary factors limiting growth and survival are expected to change through ontogeny (Houde, 1987). In this study, we performed independent tests with pre- and post-flexion larvae due to previous observations of stage-specific sensitivity to both high CO_2 (Hurst et al., 2016) and prey limitation (Suneetha et al., 1999). These experiments further demonstrate the need to consider stage-specific environmental influences as the effects of both prey limitation and elevated CO_2 levels differed between the pre- and post-flexion stages of northern rock sole. Prey restriction had a greater impact on the growth rates of post-flexion fish (43% decline in G_W from high food treatment) than pre-flexion fish (30% decline in G_W). This stage-specific difference may be related to the energetic demands of flatfish metamorphosis occurring in post-flexion larvae.

Most of reports reduced survival of marine fish larvae at elevated CO_2 levels have come from studies incorporating the earliest larval stages (although some experiments extended into later stages; Baumann et al., 2012; Pimentel et al., 2014; Frommel et al. 2014). Such an effect was observed in a previous study of northern rock sole larvae where Hurst et al. (2016) observed a trend of decreasing survival at higher CO_2 levels. In this study we observed a similar trend of higher survival at ambient CO_2 levels, but in this case the effect was only observed in the pre-flexion experiment. In the post-flexion experiment, we observed equivalent or higher survival survival rates in the high CO_2 treatment. In addition, exposures of northern rock sole eggs to elevated CO_2 levels did not lead to reduced hatching rates (Hurst et al. 2016). Together, these results may suggest that the effects of elevated CO_2 level on survival of northern rock sole larvae are primarily restricted to the earliest larval stages.

The negative effects of elevated CO_2 levels on growth and condition of post-flexion northern rock larvae observed by Hurst et al. (2016) were not similarly observed in this study (although it should be noted that the high CO_2 treatment of 1171 µatm in this study is lower than the 1541 µatm used in the previous study). In contrast, this study found that larvae reared at

elevated CO_2 levels had a slightly, but not-significantly, reduced growth rate compared to ambient controls during the pre-flexion stage and a non-significantly increased growth rate during the post-flexion stage (Fig. 2). These contrasting results between studies could be the result of genetic differences among the groups of tested fish. But in general, we believe that these differences are associated with the general observation that the effects of elevated CO_2 levels are relatively modest in this species (compared to other aspects of the rearing environment). However, this variation in outcomes also demonstrates the value of conducting multiple, independent experiments to evaluate the effects of ocean acidification and the need for caution in broad application of results across populations or species (Ferrari et al., 2011; DePasquale et al., 2015).

Experimental investigation of the effects of ocean acidification on marine organisms has broadened rapidly to include potential interactions with co-occurring environmental stressors such as elevated temperatures (Harvey et al., 2013) and hypoxia (DePasquale et al., 2015). However, it should be noted that, to our understanding, all previous experiments with early life stages of marine fishes have been conducted under nutritional conditions expected to result in high (or optimal) growth and survival in ambient control treatments. While the potential importance of nutritional conditions on the organismal response to acidification has been demonstrated in multiple invertebrate species (Drenkard et al., 2013; Hettinger et al., 2013; Towle et al., 2015), this study is the first to integrate ocean acidification with nutritional stress in a marine fish (but see Bignami 2015).

The foraging environment that larvae encounter will also be impacted by broad-scale aspects of climate change including warming and acidification. Of particular importance to the survival and growth of many commercially important fish populations will be the annual timing and magnitude of the spring bloom and associated secondary production of microzooplankton, the primary prey for most marine fish larvae. Production in these lower trophic levels is driven by a complex set of physical and biological forcing mechanisms which vary at a variety of scales and may be undergoing long-term changes associated with acidification and warming. Increased warming of surface waters is generally predicted to increase stratification, resulting in earlier and more intense primary production blooms (Rost et al., 2008; Kristiansen et al. 2011). However, such changes in timing will not necessarily result in more favorable foraging environments for newly hatched fish larvae. For example, in the Bering Sea, warm temperatures actually result in a delay in the spring bloom as the lack of sea ice cover (and the meltwater) delays stratification in

414 this light-limited ecosystem (Hunt and Stabeno, 2002). Recent years of high temperature and
415 reduced sea ice cover have resulted in later spring blooms. Variation in thermal conditions has
416 also been shown to influence the composition of the zooplankton community, presenting another
417 variable in the foraging environment for larval fishes (Hooff and Peterson, 2006; Eisner et al.,
418 2014).

419 The overall responses of lower trophic levels to ocean acidification remain unclear due to
420 the wide range of taxonomic and life history diversity in these communities. Several experiments
421 have suggested that increased CO_2 levels would be favorable for some, but not all, primary
422 producers (King et al., 2015). However, the foraging environment of most marine fish larvae will
423 be dependent on the conversion of that primary production into biomass of their zooplankton
424 prey. Experiments with zooplankton species have observed a range of effects, in some cases
425 varying across life stages within a species. Aragonite shell-forming pteropods are expected to be
426 among the most sensitive zooplankton groups to acidification (Comeau et al., 2010) along with
427 the early pelagic larval stages of bivalves and crabs. Copepods and krill are primary energetic
428 pathways in high latitude seas and serve as critical forage base for young marine fishes. Several
429 experiments have suggested that although high CO_2 exposures had minimal effects on adult
430 copepods, negative effects were observed in early life stages (Cripps et al., 2015). While there
431 have yet been few experiments with krill, negative effects of elevated CO_2 have been observed
432 (Sperfeld et al., 2014). The range of responses among co-occurring species has resulted in a
433 range of effects in community-level experiments (Rose et al., 2009; Aberle et al., 2013).
434 However, even if ocean acidification does not directly result in reduced production and
435 availability of zooplankton prey, the currently observed levels of variation due to other
436 environmental influences would suggest that fish living under future elevated levels of CO_2
437 would be expected to experience episodic, if not persistently degraded foraging conditions.

438 As species are known to respond differently to the physiological effects of elevated CO_2,
439 we similarly expect species to be differentially impacted by climate-associated changes in the
440 foraging environment. By adopting standardized prey treatments used in previous experiments
441 we were able compare the influence of reduced feeding conditions across life stages and species.
442 Northern rock sole and Pacific cod exhibit similar patterns in reproductive timing and
443 distributions of early life stages in the Gulf of Alaska (Matarese et al., 2003), but markedly
444 different growth rates during early larval stages (Hurst et al., 2010; Laurel et al., 2014). Due to
445 the overall higher demands for prey associated with fast growth, we would have expected the

more rapidly growing Pacific cod to be more sensitive to depressed feeding conditions. However, this did not appear to be the case: a similar reduction in foraging conditions resulted in only an 8% decrease in G_W of pre-flexion Pacific cod larvae (Laurel et al., 2011), compared to the 30% reduction observed in northern rock sole. The differential effects among species and life stages in response to depressed foraging conditions has important implications for the recruitment of wild fish populations in the North Pacific under shifting environments.

Conclusions

The results of these experiments support the observations from previous work examining the potential impacts of climate change on the growth and survival of north Pacific marine fishes. As observed in earlier work on walleye pollock and northern rock sole, elevated CO_2 levels resulted in only minor impacts on growth and survival (Hurst et al., 2013; 2016). In this first test with a marine fish, diminished foraging conditions did not appear to induce stronger responses to elevated CO_2. However it should be noted that experiments with other species have revealed clear, negative, direct physiological effects of elevated CO_2 (Baumann et al., 2012; Frommel et al., 2014; Pimentel et al., 2014), and it might be expected that a poor nutritional environment could exacerbate the observed effects in more CO_2-sensitive species. Conversely, the foraging environment encountered by northern rock sole larvae had a substantial impact on the growth and development rates of both pre-flexion and post-flexion stages. Other environmental variables are also known to impact larval dynamics. For example, in both laboratory experiments and field studies, the time required for rock sole larvae to reach metamorphosis and settle to demersal juvenile habitats has been shown to be affected by water temperature (Laurel et al., 2014; Fedewa et al., 2015). These results suggest that for some species, factors other than the direct, energetic effects of acidification are most likely to determine population productivity under future climate conditions.

It is important to recognize that this study examined only energetic aspects of the response to ocean acidification in this species. Numerous experiments have indicated that physiological responses to hypercapnia may disrupt the transmission of nerve signals (Nilsson et al., 2012), altering the behavioral responses to sensory stimuli (Leduc et al., 2013). It is possible that these behavioral or sensory deficits could significantly impair prey recognition and capture, with ocean acidification resulting in a physiologically-induced diminished foraging ability in wild fish. Alternatively, ocean acidification, in conjunction with other aspects of anthropogenic

478 climate change, could negatively impact the foraging conditions faced by larval fishes, ultimately
479 impacting population production. Continued research on the independent and interactive effects
480 of acidification on the primary and secondary producers that serve as a forage base for larger
481 organisms will be critical to predicting how changes in the lower trophic levels will propagate
482 through the food web, influencing the fisheries of the world.
483
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492
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Table 1. Conditions during experimental exposures of northern rock sole (*Lepidopsetta polyxystra*) eggs and larvae to projected ocean acidification. Carbonate system parameters (dissolved inorganic carbon, DIC; total alkalinity, TA; temperature; and salinity) were measured 2-3 times per week and used to calculate pH and pCO_2. Values shown are means ± 1 std. dev.

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Experiment-Treatment	Temp. (°C)	DIC (µmol kg^{-1})	TA (µmol kg^{-1})	pH (seawater scale)	pCO_2 (µatm)
Parental broodstock	5.1 ± 0.5	2011.2 ± 56.1	2127.2 ± 59.0	7.99 ± 0.08	415 ± 76
Trial 1 – pre-flexion larvae					
Ambient	7.1 ± 0.5	2016.3 ± 74.8	2160.8 ± 82.6	8.07 ± 0.07	349 ± 60
High CO_2	7.1 ± 0.5	2137.1 ± 76.1	2132.1 ± 70.1	7.63 ± 0.13	1079 ± 396
Trial 2 – post-flexion larvae					
Ambient	7.4 ± 0.8	2043.3 ± 92.5	2173.4 ± 69.1	8.01 ± 0.18	453 ± 213
High CO_2	7.4 ± 1.0	2182.5 ± 67.2	2183.8 ± 64.0	7.59 ± 0.10	1171 ± 277

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670 **Table 2.** Analysis of variance of size condition factors of pre-flexion northern rock sole
671 (*Lepidopsetta polyxystra*) larvae as a function of feeding regime and CO_2 level. Response
672 variables are the tank mean value measured on each sampling date in each of the replicate
673 rearing tanks.
674

Size		Length (SL)		Body depth (MH)		Dry weight (DW)	
	d.f.	F	*p*	F	*p*	F	*p*
Age	3	363.1	< **0.001**	263.5	< **0.001**	254.5	< **0.001**
CO_2 level	1	1.6	0.212	0.7	0.393	0.4	0.513
Prey level	2	77.0	< **0.001**	60.6	< **0.001**	58.7	< **0.001**
Age x CO_2	3	0.21	0.891	< 0.1	0.995	< 0.01	0.988
Age x prey	6	9.0	< **0.001**	10.7	< **0.001**	12.2	< **0.001**
CO_2 x prey	2	0.2	0.835	0.2	0.789	0.4	0.701
Error	44						

Condition Factor		MH_{dev}		DW_{dev}	
	d.f.	F	*p*	F	*p*
Age	3	6.5	< **0.001**	5.5	< **0.001**
CO_2 level	1	< 0.1	0.932	0.7	0.392
Prey level	2	6.9	< **0.001**	0.2	0.836
Age x CO_2	3	1.8	0.157	2.4	0.080
Age x prey	6	11.3	< **0.001**	3.7	< **0.001**
CO_2 x prey	2	1.55	0.223	0.5	0.586
Error	44				

Table 3. Analysis of variance of size condition factors of post-flexion northern rock sole (*Lepidopsetta polyxystra*) larvae as a function of feeding regime and CO_2 level. Response variables are the tank mean value measured on each sampling date in each of 3 replicate rearing tanks in each experiment.

Size		Length (SL)		Body depth (MH)		Dry weight (DW)	
	d.f.	F	*p*	F	*p*	F	*p*
Age	3	304.5	< **0.001**	600.1	< **0.001**	165.3	< **0.001**
CO_2 level	1	5.5	0.025	1.42	0.393	0.5	0.502
Prey level	1	506.6	< **0.001**	932.9	< **0.001**	361.0	< **0.001**
Age x CO_2	3	0.2	0.928	0.2	0.995	1.2	0.308
Age x prey	3	21.3	< **0.001**	102.9	< **0.001**	60.2	< **0.001**
CO_2 x prey	1	4.4	**0.044**	3.5	0.789	0.6	0.429
Error	35						

Condition Factor		MH_{dev}		DW_{dev}	
	d.f.	F	*p*	F	*p*

Age	3	5.1	**0.004**	5.5	**0.003**
CO_2 level	1	7.7	**0.009**	0.9	0.349
Prey level	1	10.9	**0.002**	1.3	0.254
Age x CO_2	3	0.4	0.741	.06	0.595
Age x prey	3	10.0	< **0.001**	3.4	**0.029**
CO_2 x prey	1	1.2	0.280	0.4	0.533
Error	35				

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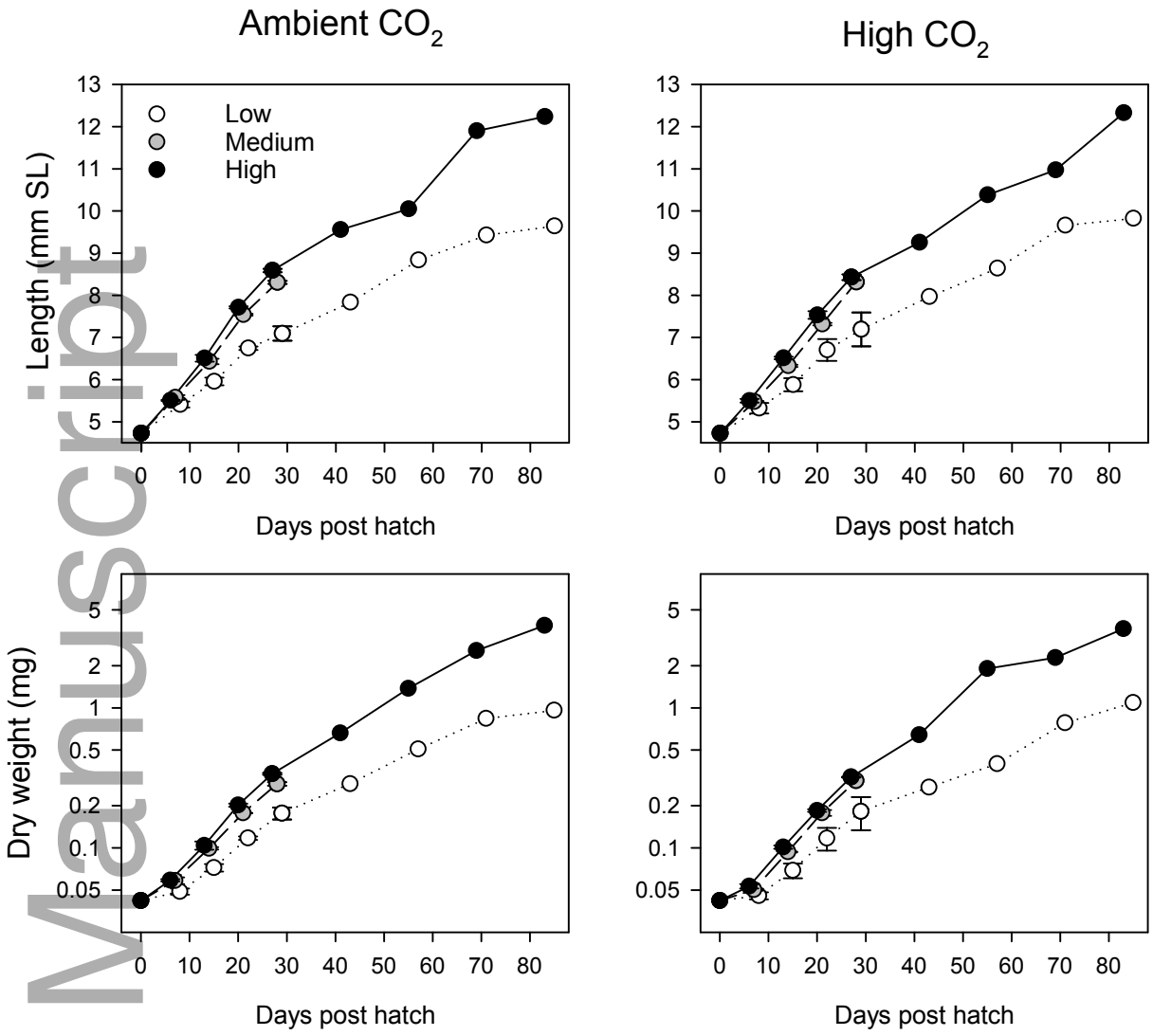
683
684 Fig. 1. Length and weight of pre-flexion northern rock sole (*Lepidopsetta polyxystra*) larvae
685 reared at ambient and elevated CO_2 levels at three prey densities. Points represent mean (± se) of
686 two-three replicate tanks within each treatment through 31 DPH. For measurements at 45-87
687 DPH, points are mean fish size in one tank at each treatment. Note: all tanks sampled on the
688 same days, points offset horizontally for clarity.
689
690 Fig. 2. Growth rates (± se) in length and mass of northern rock sole (*Lepidopsetta polyxystra*)
691 larvae as a function of CO_2 level, feeding regime, and developmental stage.
692
693 Fig. 3. Abundances and developmental stages of northern rock sole larvae (*Lepidopsetta*
694 *polyxystra*) larvae reared as a function of CO_2 level prey density. Top: mean abundances within
695 treatment averaged across replicate rearing tanks within each treatment at 31 DPH; Bottom,
696 abundance in one tank at each treatment at 87 DPH.
697
698 Fig. 4. Length and weight of post-flexion northern rock sole (*Lepidopsetta polyxystra*) larvae
699 reared at ambient and elevated CO_2 levels and high and low prey densities. Points represent
700 mean (± se) of three replicate tanks within each treatment. Note: all tanks sampled on the same
701 days, points offset horizontally for clarity.
702
703 Fig. 5. Abundances and developmental stages of northern rock sole larvae (*Lepidopsetta*
704 *polyxystra*) larvae reared at ambient and elevated CO_2 levels at two prey densities from 31 to 87

705 DPH. Values are mean abundances within treatment averaged across three replicate rearing
706 tanks.



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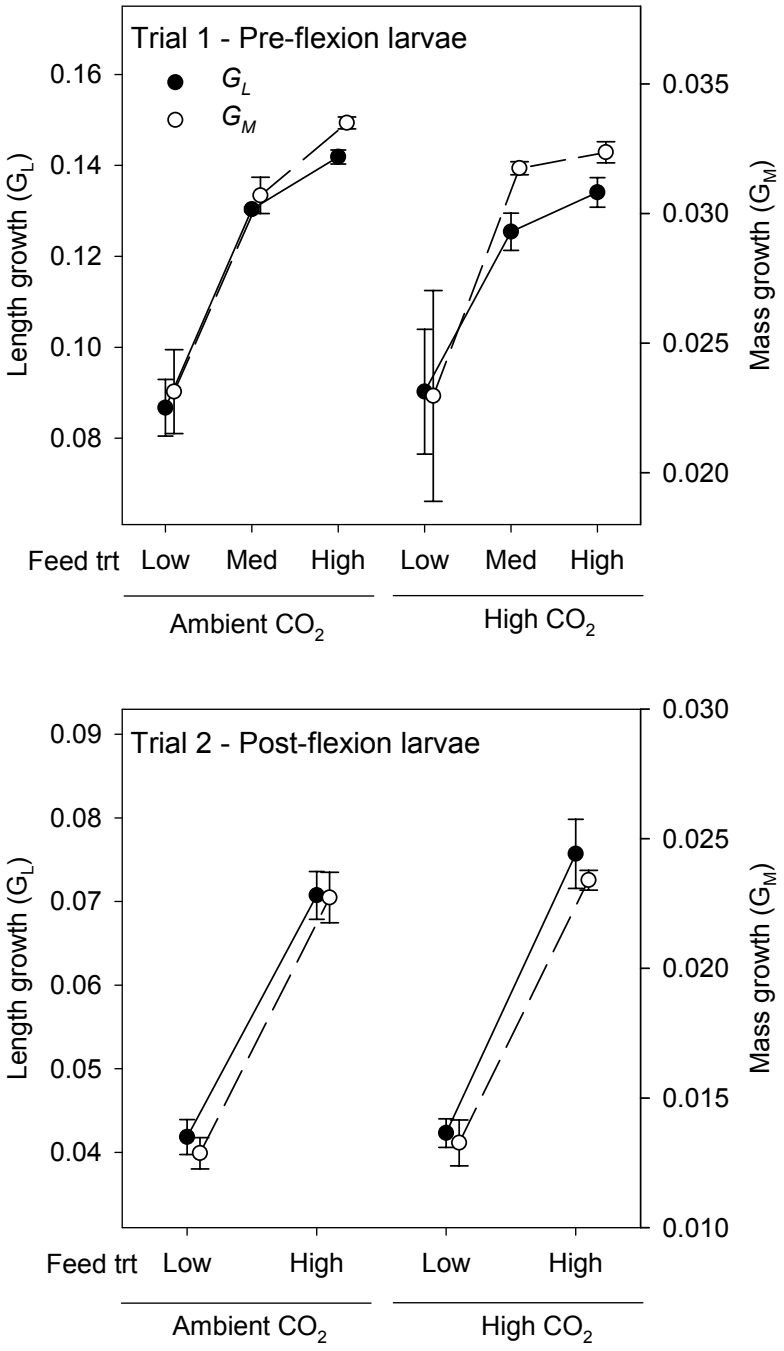

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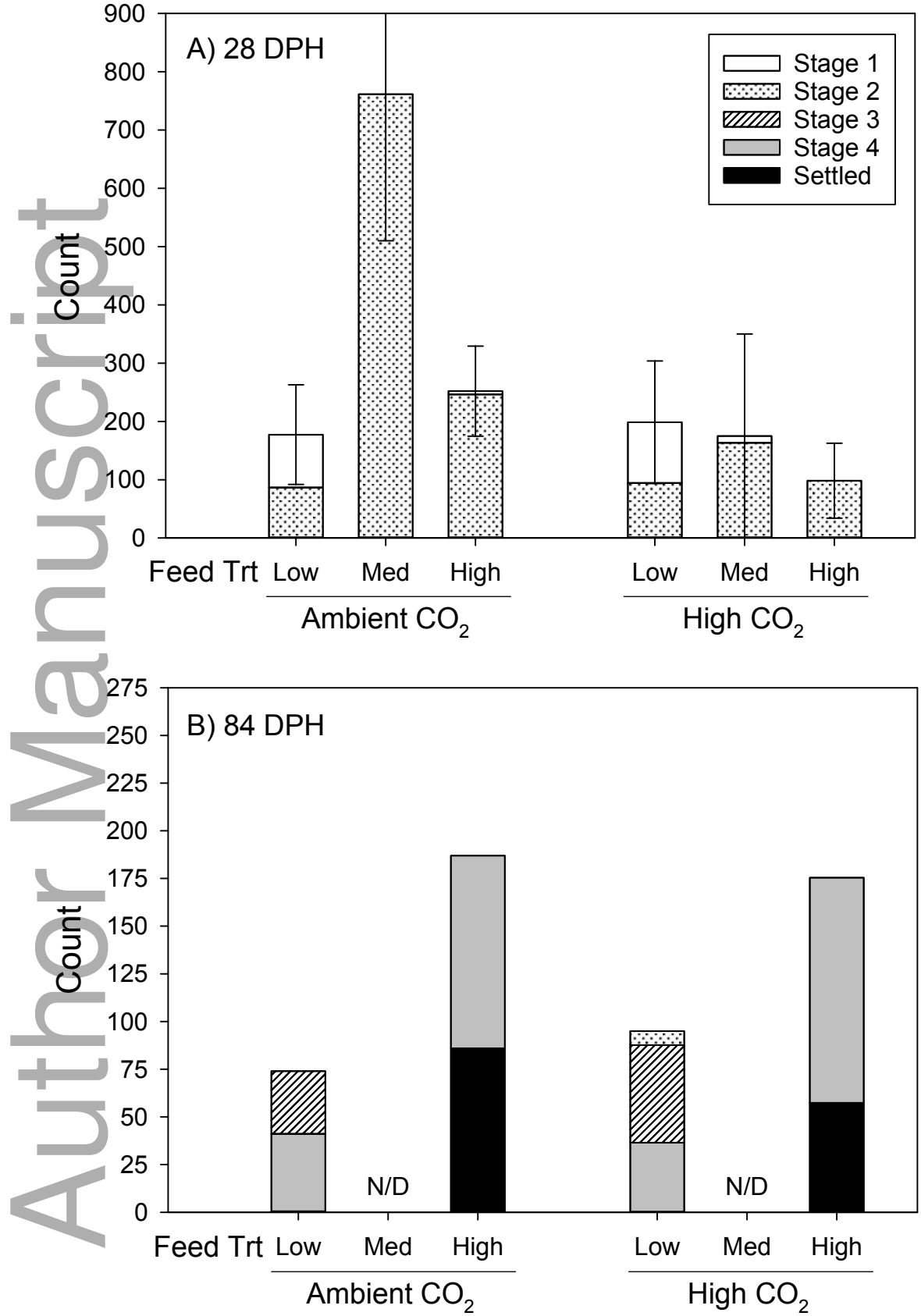

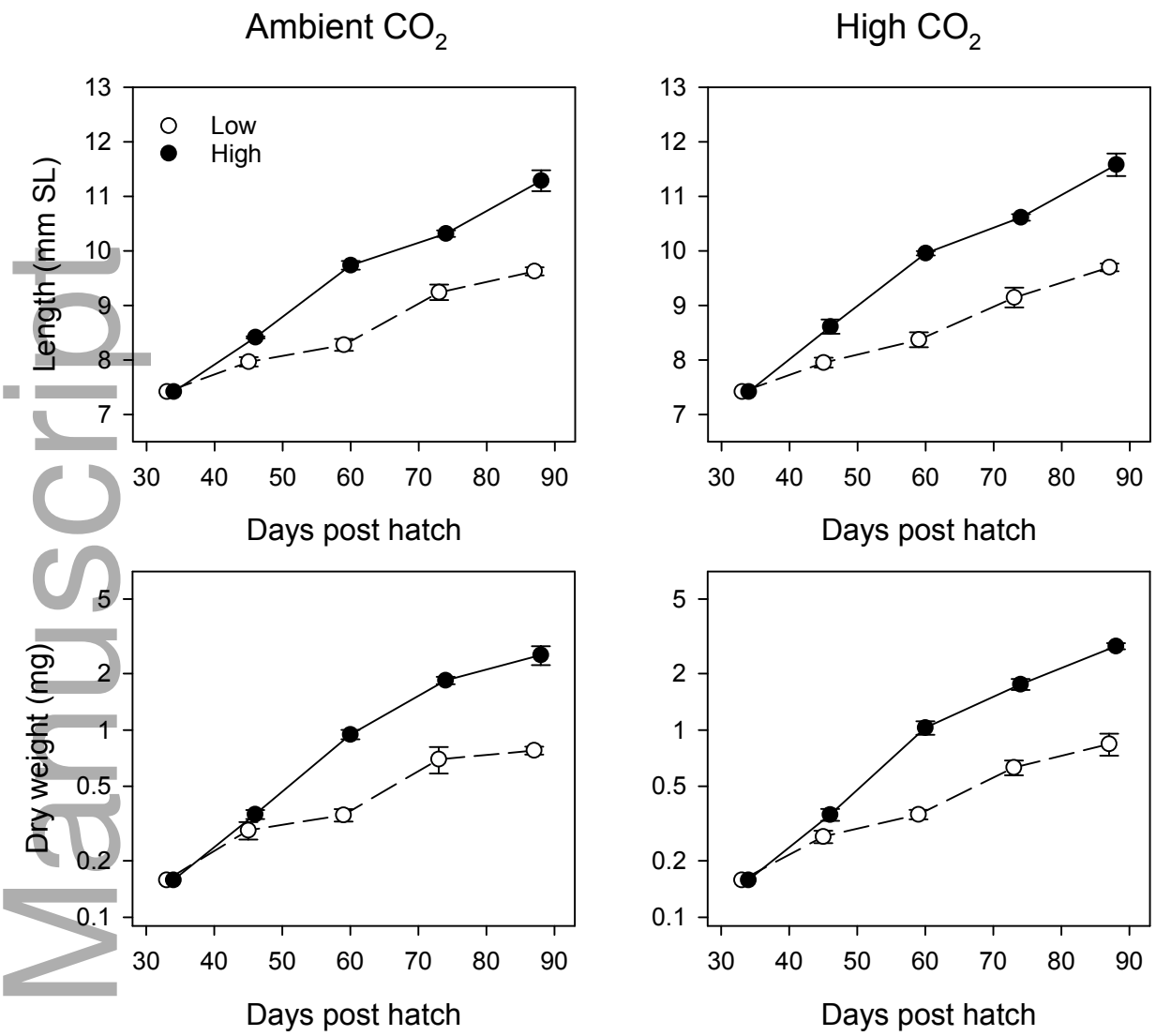

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