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Divergent responses of Atlantic cod to ocean acidification and food limitation

Running title: Acidification affects growth of cod larvae

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Abstract

In order to understand the effect of global change on marine fishes, it is imperative to quantify the effects on fundamental parameters such as survival and growth. Larval survival and recruitment of the Atlantic cod (*Gadus morhua*) was found to be heavily impaired by end-of-century levels of ocean acidification. Here, we analysed larval growth among 35-36 days old surviving larvae, along with organ development and ossification of the skeleton. We combined CO_2-treatments (ambient: 503 *µ*atm, elevated: 1179 *µ*atm) with food availability in order to evaluate the effect of energy limitation in addition to the ocean acidification stressor. As expected, larval size (as a proxy for growth) and skeletogenesis were positively affected by high food availability. We found

significant interactions between acidification and food availability. Larvae fed ad libitum showed little difference in growth and skeletogenesis due to the CO_2 treatment. Larvae under energy limitation were significantly larger and had further developed skeletal structures in the elevated CO_2 treatment compared to the ambient CO_2 treatment. However, the elevated CO_2 group revealed impairments in critically important organs, such as the liver, and had comparatively smaller functional gills indicating a mismatch between size and function. It is therefore likely that individual larvae that had survived acidification treatments, will suffer from impairments later during ontogeny. Our study highlights important allocation trade-off between growth and organ development, which is critically important to interpret acidification effects on early life-stages of fish.

Introduction

Over the last 150 years anthropogenic activities have led to an increase in atmospheric concentrations of carbon dioxide, which may increase globally to 750-1000 ppm CO_2 by 2100 under current climate projections (i.e. RCP 8.5 (IPCC, 2013)). Significant effects on marine ecosystems are to be expected through changes in ocean acidification levels (direct effects) and increases in temperature via the indirect effects of CO_2 as a potent greenhouse gas (Doney, Fabry, Feely, & Kleypas, 2009; Orr et al., 2005). Many commercially and ecologically important fish populations are progressively subjected to these environmental changes with effects at all biological levels already visible now. Owing to the higher solubility of CO_2 in colder waters an even sharper increase in ocean acidification levels in these high latitude regions is predicted (Steinacher, Joos, Frölicher, Plattner, & Doney, 2009). A thorough understanding of these effects on individuals and fish populations becomes

increasingly important, particularly for species that are commercially exploited and where management may need adjustment under consideration of global change effects (Denman, Christian, Steiner, Pörtner, & Nojiri, 2011; Kjesbu et al., 2014; Lam, Cheung, & Sumaila, 2014).

Studies on the effects of ocean acidification on marine fishes, especially the early life stages that are most vulnerable to ocean acidification, have steadily increased in recent years and revealed different effects. Such a pronounced interspecies variability is possibly due to different experimental setups, different analyzed life stages (often limited to very early life stages, e.g. embryos, non-feeding larvae), and short-term versus long-term effects of the applied treatments. For example, no effect on growth rate was observed for walleye pollack (*Theragra chalcogramma*) (Hurst, Fernandez, & Mathis, 2013), juvenile scup (*Stenotomus chrysops*) (Perry et al., 2015) and the larvae of yellowtail kingfish (*Seriola lalandi*) (Watson et al., 2018). For other species a decrease in growth rate under elevated CO_2 was observed, e.g. in the inland silverside (*Menidia beryllina*) (Baumann, Talmage, & Gobler, 2012), for yellowfin tuna (*Thunnus albacares*) (Bromhead et al., 2015), and Atlantic herring (*Clupea harengus*) (Frommel et al., 2014). However, another study found no effect on Atlantic herring (Sswat, Stiasny, Jutfelt, Riebesell, & Clemmesen, 2018) . Still others showed an increase in growth rate e.g. in orange clown fish (*Amphiprion percula*) (Munday, Donelson, Dixson, & Endo, 2009), in cod (Frommel et al., 2012) in summer flounder (*Paralichthys dentatus*) (Chambers et al., 2013, 2014) and in sand smelt larvae (*Atherina presbyter*) (Silva et al., 2016) under elevated pCO_2 levels.

Most of the studies on the effect of ocean acidification on larval fishes have been using ad libitum or generally high food densities that are unrealistic in nature. Only recently studies have taken the possible interaction of CO_2 level and food supply into consideration, assuming that ad libitum food availability could potentially compensate for any direct effect of CO_2 on survival, growth and development (Gobler, Merlo, Morrell, & Griffith, 2018; Hurst, Laurel, Hanneman, Haines, & Ottmar, 2017; Sswat, Stiasny, Taucher, et al., 2018; Stiasny et al., 2018). Kreiss *et al.* (2015) have hypothesized that ocean acidification increases costs for osmoregulation and baseline energy demands in adult cod. A limited energy uptake due to lower food availability may therefore be required for ocean acidification effects to become experimentally visible. For example, in juvenile blue mussels, food availability outweighed the negative effects of ocean acidification (Thomsen, Casties, Pansch, Körtzinger, & Melzner, 2013) and an increase in feeding rate in corals was able to mitigate the negative effects of ocean acidification on calcification (Towle, Enochs, & Langdon, 2015). We therefore wanted to test ocean acidification effects in combination with food limitation.

Knowledge on the bioenergetics of development under ocean acidification and how organisms prioritize endogenous energy flow toward development and physiological function is mostly unknown (Matson, Yu, Sewell, & Hofmann, 2012). It is increasingly important to understand the underlying mechanisms of CO_2 effects on growth and how energy limitation may lead to important yet poorly understood possible metabolic trade-offs. We have therefore chosen to include RNA/DNA ratio analyses in this study to determine larval condition. RNA/DNA ratios, indicators of protein biosynthesis machinery, can reflect the nutritional condition of larval fish (Clemmesen, 1994; Buckley *et al.*, 2008;) and have been used to reflect changes in energy availability in relation to ocean acidification (Franke & Clemmesen, 2011; Frommel et al., 2014) and other environmental stressors (Chícharo & Chícharo, 2008).

Frommel *et al.* (2012) suggested that the increase in body mass of cod larvae under experimental ocean acidification was mostly caused by an increase in lipid content observed under elevated CO_2 levels and indicated an altered pathway in lipid and protein metabolisms. Díaz-Gil *et al.* (2015) observed shifts in the relative proportions of fatty acids in the tissues of red drum and Silva *et al.* (2016) showed a higher cellular energy consumption rate at elevated CO_2 levels in sand smelt measured by changes in enzyme levels. A possible reason for the sensitivity of these larval stages to ocean acidification might be the functional development of organs such as the gastro-intestinal tract (Zambonino Infante & Cahu, 2001), the gills and the urinary system with their osmoregulatory functions (Varsamos, Nebel, & Charmantier, 2005). Yet, research on the functional development under the influence of ocean acidification in fish larvae is still limited. Hence one of the key objectives of this study was to determine both, growth parameters, including lipid content, as well as developmental organ and skeletal differences. So far, only limited information is available how ocean acidification effects these areas of development (Frommel et al 2012, Pimentel *et al.*, 2014a; Crespel *et al.*, 2017).

We studied how the trade-offs among growth and development under combined ocean acidification and food limitation played out in Atlantic cod (*Gadus morhua*), one of the most important commercial species in the North Atlantic (FAO, 2016). Larvae of the North-East Arctic cod stock showed a doubling of mortality rates after hatching under end-of-the-century acidification levels with marked negative effects when modelling subsequent population recruitment (Koenigstein et al., 2018; Stiasny et al., 2016). Here we assessed whether the surviving larvae (35 to 36 dph) subjected to ambient and elevated CO_2 treatments (503 +/- 89 and 1179 +/- 87 µatm pCO_2, 7.9 and 7.61 pH, respectively) were affected in their growth and development, by explicitly considering the nutritional status of the larvae in a fully crossed

design. The chosen level of ocean acidification falls within the possible scenarios of climate change in the Arctic (AMAP, 2013). We compared responses of larvae fed different amounts of prey in a 2 x 2 factorial design to include the effect of energy limitation. Single and interactive effects were estimated on various growth and condition parameters, as well as skeletal and organ development. We discuss the potential trade-offs between growth and development to these different acidification levels and feeding regimes.

Materials and methods

The experiment was performed in 2014 at the Norwegian National Cod Breeding Centre (recently renamed to Centre for Marine Aquaculture), in Tromsø, Norway. In order to obtain eggs and larvae, adult cod were caught alive in the Barents Sea at roughly 70°15'N, 19°00'E in March 2014 and transferred to the Centre in Tromsø. They were kept in large breeding tanks (25 m^3) at ambient temperature, light and CO_2 conditions. Spawning occurred naturally two weeks after introduction into the breeding tanks and all eggs were collected from the outflow. The fertilized eggs were transferred to incubators with either ambient (503 ± 89 μatm CO_2) or increased CO_2 (1179 ± 87 μatm) concentrations. Egg developmental stages were examined under a stereomicroscope to ensure that several females participated in these spawning events (Fridgeirsson, 1978). Three different egg developmental stages indicating different spawning times during the night, which cannot be produced by the spawning event of one female, were observed. This result indicates that at least three different females had participated at the spawning event, since a single female will not spawn multiple times within one day (Kjesbu, 1989). At 100% hatch, occurring at 112 degree-days, 11,000 larvae were transferred into each of the twelve 190 L rearing tanks with a constant flow-through of water from two header tanks. Six tanks each were supplied from a header tank with ambient water

and from a header tank containing water with increased CO_2 concentrations. For the egg incubation and the start of the experiment the temperature was set to 6°C and was later, in the larval tanks, raised to 10°C simultaneously in all tanks, while kept at constant light conditions (24h light). Larvae were fed with *Nannochloropsis* (until day 12 days post-hatching (dph)) and *Brachionus* until 24 dph, after which feeding was gradually shifted to *Artemia* nauplii. Prey concentrations fed at each feeding were the same for both treatments, but number of feedings and therefore total amount fed per day differed (Supplement S Table 1). Though prey availability in this set-up is hard to compare to natural conditions, since larvae were pulse-fed with high concentrations of prey, it is likely that the energy limitation, even in the low food treatment, is still above most conditions encountered by larvae in the field.

Larvae in one tank in the ambient CO_2 treatment were abruptly lost over night, due to an unknown factor, resulting in six replicates for the elevated CO_2 treatment and five for the ambient treatment. This study was carried out in strict accordance with the laboratory regulations applicable in Norway. The application was approved by the National Regulatory Committee on the Ethics of Animal Experiments (Permit FOTS id 6382). All conditions and samplings were conducted to minimize suffering.

The values in the ambient CO_2 treatment were pCO_2: 503 ± 89 µatm; pH 7.9 ± 0.15 and pCO_2: 1179 ± 87 µatm; pH 7.61 ± 0.03 levels in the elevated CO_2 rearing tanks were achieved by controlling the pH values in a header tank with pH probes connected to an IKS computer system. The volume of the header tank ensured a thorough mixing and equilibration of CO_2 before the water entered the rearing tank thereby assuring constant conditions in the rearing tanks. The pH and temperature were furthermore manually checked

every day in all the rearing tanks with a separate pH/temperature probe (WTW pH/Cond 340i/3320). Water chemistry, including DIC and alkalinity, was measured weekly. For further details please consult Stiasny *et al.* (2016) or Stiasny *et al.* (2018).

For the growth measurements larvae at 36 dph were sampled alive, euthanized with MS222 (Ethyl 3-aminobenzoate methanesulfonate) and frozen at -20°C (N= 11-27 per treatment). The sampled larvae were later photographed under a stereomicroscope next to a micrometer scaling bar. The photographs were then used to measure the larval sizes using the software ImageJ. In order to measure dry weight, larvae were freeze-dried (Christ Alpha 1-4 freeze dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany) before being weighed (Sartorius SC2 microbalance, Sartorius AG, Göttingen, Germany, precision 0.1µg).

For the ossification analysis, larvae at 36 dph were fixed in 70% ethanol (Schnell, Konstantinidis, & Johnson, 2016). For the investigation of the skeletal development, the larvae were cleared and double stained (c&s) in an acid-free c&s method, following a modified protocol of Walker & Kimmel (2007). After fixation specimens were stained for cartilage in 6 ml of an acid-free alcian blue staining solution corresponding to Part A of Walker & Kimmel (2007). After 24 h the specimens were washed in a 70% ethanol solution to remove excessive alcian blue and transferred for another 24 h into 6 ml of a 0.5% KOH solution containing four droplets of a 3% H_2O_2 solution and alizarin red powder. Tissues of the larvae were then cleared by KOH, bleached by H_2O_2 and stained for calcified structures by alizarin red. Finally specimen were transferred to a 70% glycerin solution for dissection and digital documentation. All specimen were digitally photographed with an Axiocam microscope camera attached to a ZEISS Discovery V20 stereomicroscope and processed with

the Zeiss ZEN software. The number of ossified vertebrae of between seven to 19 larvae per treatment was counted on these photographs. To measure the gill structures seven to twelve larvae per treatment were used. The branchial basket of each larva was removed and the third ceratobranchial was dissected out and photographed in order to measure the ossified structure (the pink stained structure in Figure 1) in length (horizontal line in Figure 1a), the length of the longest gill filament (vertical line in Figure 1a) and the area of gill filaments above the ossified part of the ceratobranchial (as shown in red in Figure 1b).

For the histological analysis, four to nine larvae per treatment were fixed in 4% buffered formaldehyde at 35 dph, embedded in *Technovit*® or paraffin, sectioned transversely or longitudinally at 3µm, followed by staining with methylene blue or haematoxylin and eosin respectively. *Technovit*-sections from head region (with eyes, gills and heart), front part of gut (with liver, pancreatic tissue, kidney tissue) as well as paraffin sections were studied and photographed with the microscope (Leitz Aristoplan with a Leica DFC295 camera). Moderate or numerous amounts of vacuoles in the pigment layer of the retina were noted and given a 4-category, qualitative score (normal 0/ some +/ several ++/ many +++). A similar score was used for registration of lipid vacuoles in the cod larvae livers.

Prior to RNA/DNA analyses and lipid determination all larvae previously stored at -80°C were freeze dried for 16 h and weighed (precision 0.1 µg). Measurements of RNA and DNA were made using the fluorimetric method described by Clemmesen (1993) and modified by Malzahn *et al.* (2007). Five larvae from three replicate tanks each were freeze dried, weighed and the tissue was homogenized in 400 µl or 800 µl 0.01% sodiumdodecyl sulfate Tris buffer

(TE SDS), depending on the individual larval dry mass. When necessary, the homogenate was diluted up to 10-fold with 0.01% TE-SDS prior to fluorimetric determination. Ethidium bromide was used as a specific nucleic acid fluorescent dye for both RNA and DNA, and the total fluorescence was measured (Fluoroskan Ascent, Thermo Scientific, Waltham, Massachusetts, USA). RNAse was then used to digest all RNA enzymatically. The RNA fluorescence was calculated by subtracting the DNA fluorescence from the total fluorescence. By using the calibration curve fitted to the standard measurements (23 s r-RNA Boehringer, Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) the amount of RNA was calculated. Following Le Pecq & Paoletti (1966), the DNA concentration was calculated using the relationship between RNA and DNA fluorescence with a slope ratio of standard DNA to standard RNA of 2.2, which adjusts for the relative fluorescence intensity difference of RNA and DNA.

Total lipids were extracted from individual freeze-dried and weighted cod larvae (five additional larvae from each tank) using a modification of the Folch method (Folch, Lees, & Stanley, 1957) with dichloromethane/methanol/chloroform (1:1:1 v/v/v). Freeze dried larvae were individually placed in 1.5 ml of the solution in a glass vial, securely capped with Teflon lined screwcaps and stored at -80°C for 72 h. The defatted carcasses were transferred to Eppendorf vials and placed with open lids in a desiccator for 48 h to allow for evaporation of the remaining lipid solvents before determining the defatted dry weight on a microscale (Sartorius SC2 microbalance). By subtracting the dry weight of the defatted carcass from the total larval dry weight, the amount of lipids could be gravimetrically determined and presented as lipids in % of dry weight.

For the analyses linear mixed-effects models were used to test for interactions between the CO_2 and the food treatment (as fixed effects) and their effect on larval size, while using the tank ID as a random effect. In case of the gill and the vertebrae measurements, standard length was included as a covariate to account for differences in gill size or stage of ossification that could be due to larval size rather than CO_2 treatment. The model always followed the following formula: model <- lmer (Parameter ~ CO_2 treatment * Food treatment [*standard length] + (1 | Tank ID). No assumptions of this analysis were violated. Lipid content was logit transformed as recommended for proportional data (Warton & Hui, 2011). No other parameters needed to be transformed. P-values were later obtained using the anova(model) function.

All statistical analyses were run in the programs R (Version 3.3.2) and RStudio (Version 1.0.136). Graphics were done in the R package ggplot2 (Wickham, 2009) and the linear mixed-effects model was run in the R package "lme4" (Bates, Mächler, Bolker, & Walker, 2015).

Results

The CO_2 and food treatment showed a significant interaction effect on larval size, both in terms of standard length (SL, t=-3.49, p<0.001) as well as dry weight (DW, t=-2.021, p=0.043) (All results of the statistical analyses are shown in detail in Supplement S Table 2). Larvae in the high-food treatment were significantly longer (t=-3.97, p<0.0001) and heavier (t=-8.521, p<0.0001) than those in the low-food treatment. While larval size at the high-food regime was not affected by the acidification, larvae at the low-food regime were heavier

(mean: 0.49mg vs. 0.29mg) and longer (mean: 8.9mm vs. 7.8mm) under elevated CO_2 compared to ambient CO_2 levels (Figure 2). The variance due to the tank ID was consistently small in all parameter's analyses.

The number of ossified vertebrae increased linearly with the standard length in all treatments (Supplement S Table 2, Figure 3). After taking this relationship into account, both CO_2 and food treatment had a strong interactive effect on the number of vertebrae that were fully ossified ($t=2.054$, $p<0.0001$). Under ambient CO_2, larvae in the high-food treatment had about twice as many fully ossified vertebrae than those in the low-food treatment, both normalized for standard length. However, larvae in the low-food, elevated CO_2 treatment had more fully ossified vertebrae than those in the low-food, ambient CO_2 treatment, closely resembling the relationship between standard length and number of ossified vertebrae of the high food/ambient CO_2 treatment. In the elevated CO_2 treatment, the relationship between the number of fully ossified vertebrae and standard length was similar between high and low-food treatment larvae (Figure 3).

The RNA/DNA ratios were most strongly affected by the food treatment ($t=-10.39$, $p<0.0001$) with much higher ratios in the high food treatment. Additionally, there was an interactive effect between the CO_2 and food treatments ($t=-2.32$, $p=0.02$). In the high-food regime, RNA/DNA ratios were similar between the CO_2 treatments. However, in the low-food regime RNA/DNA ratios did differ between CO_2 regimes with 25% lower RNA/DNA ratios in the ambient treatment (Figure 4a).

The lipid content (Figure 4b) showed a strong interactive effect of the CO_2 and food treatment ($t=3.38$, $p<0.001$). It was on average between 18 and 19% in all larvae from the elevated CO_2 treatment and in those from the ambient CO_2, high-food treatment, but significantly higher in the larvae from the ambient CO_2, low-food treatment with a mean above 22%.

The length of the ossified gill arches was most strongly linked to the larval size ($t=1.905$, $p<0.0001$, Supplement S Table 2). Nevertheless, there was also a strong interactive effect of the two treatments ($t=0.931$, $p=0.006$). (Figure 5a). Under ambient CO_2 concentrations larvae in the low food treatment had shorter ossified gill arches than those in the high food treatment, even after applying standard length as a covariate. Larvae under elevated CO_2 showed no interaction with feeding level, with gill arch lengths increasing linearly with larval size.

The size of the gill filaments showed a different picture. Both filament length and gill area were strongly positively correlated to the standard length of the larvae ($t=8.75$, $p=0.02$; $t=1.764$, $p<0.0001$ respectively, Figure 5 b, c). The length of the longest gill filament was additionally affected by the CO_2 treatment ($t=-2.232$, $p<0.0001$) showing longer filaments under ambient CO_2 compared to elevated CO_2 independent of larval size or food treatment (Figure 5b). Gill area was significantly affected by the interaction of both treatments ($t=1.181$, $p<0.001$). In the ambient CO_2 treatment, larger gill areas were found in the high-food regime than in the low-food regime. In the elevated CO_2 treatment larvae showed a

similar gill area to standard length relationship independent of their own food treatment. Overall the larvae in the elevated CO_2 treatment had gill areas close to those in the ambient, low food treatment, and were smaller than those in the ambient, high food treatment (Figure 5c).

The severity of ocean acidification and food limitation induced organ damage depended on the particular organ. While vacuoles in the eyes were found across all treatments, vacuoles in the pancreas, the kidneys and particularly in the liver were most common in larvae in the elevated CO_2, low-food regime. Damage in the pancreas and the kidneys was mild, but more severe vacuolization was observed in the liver of larvae from the elevated CO_2 low-food treatment (Figure 6).

Discussion

Both prey availability and ocean acidification produced strong interactive effects on growth and development of Arcto-Norwegian cod larvae that would have remained undetected when manipulating both factors in isolation. This result is particularly relevant, since we chose a realistic end-of-the-century CO_2 concentration for the ocean acidification level (AMAP, 2013; Denman et al., 2011). Specifically, only larvae in the low-food treatment showed significant differences according to the CO_2 treatment. Here, larvae were heavier, longer, had higher RNA/DNA ratios, lower lipid contents and longer ossified gill arches compared to larvae from the ambient CO_2 treatment. This was unexpected since osmoregulation in fish is an energetically costly process, facilitated by ATPase (Melzner et al., 2009), which would suggest that the larvae from the elevated CO_2 treatment should be energetically more limited.

It is noteworthy that growth and skeletal development is similar in larvae from the high CO_2, low-food treatment and larvae from the high-food, ambient CO_2 treatment. For a quick recapitulation: larvae in the low-food, elevated CO_2 treatment had more fully ossified vertebrae than those in the low-food, ambient CO_2 treatment, and with that closely resemble the relationship between standard length and number of ossified vertebrae of the high food/ambient CO_2 treatment. This result in combination with the liver histology (see discussion below) indicates a compensatory effect in larvae, facing stressors like high CO_2 and low food, in order to maintain their growth and development. This compensatory effect might be achieved by altered energy allocation in the liver (i.e. highly vacuolized and less storage in the liver) and the lower body lipid content resulting in changed organ development/histomorphology and ossification. As a consequence particularly the liver may possibly be impaired by the vacuolization found in the low-food, elevated CO_2 treatment, which was not found in the high-food, elevated CO_2 treatment. Since our sample size for histological analysis was very low and statistical analyses were not possible, the conclusion drawn from the histological data have to be taken with caution. But similar organ impairments of liver and gills have been already documented in cod, herring, tuna and summer flounder in response to ocean acidification (Chambers et al., 2013; Frommel et al., 2012, 2014, 2016; Stiasny et al., 2018). The observed lower body lipid content in the larvae from the low food elevated CO_2 treatment might be associated with the increase in ossification, since dietary lipids are important energy stores for skeletal development in marine fishes and might have been allocated for the ossification process (Lall & Lewis-McCrea, 2007; Kjørsvik *et al.*, 2009). Additionally, high lipid levels of larvae in the low-food, ambient CO_2 treatment suggest that less energy was used for ossification and developmental processes compared to the other treatments. This hypothesis is supported by the smaller larval size and fewer ossified vertebrae.

The gill size was always smaller in the elevated CO_2 treatment, independent of food regime and larval size. Considering that the active surface of the gill is a complex three dimensional structure and therefore several fold larger than the dimensional area that was measured (Lefevre, McKenzie, & Nilsson, 2017), these differences suggest a marked impairment of gill functionality, with potentially severe effects on individual performance, including osmoregulation, oxygen uptake and swimming performance. For example, Watson et al. (2018) found that juvenile kingfish had reduced swimming performance when reared at elevated CO_2, which could be a consequence of reduced gill area as observed here. Considering the gill is also the primary organ for acid-base regulation, it poses the question whether ocean acidification effects may lead to a vicious circle in larval development. Ocean acidification may hamper gill development, leaving the young fish with even less potential to react to pH changes. There is significant need for further research looking into the effects of ocean acidification on physiology and development.

Increased calcification of the otoliths due to acidification has been shown for several species (Bignami, Enochs, Manzello, Sponaugle, & Cowen, 2013; Hurst et al., 2012; Maneja et al., 2013; Pimentel et al., 2014; Réveillac et al., 2015). This is likely due to the increased concentration of bicarbonate ions in the blood, which is elevated as a buffering mechanism during hypercapnia or pH stress (Melzner et al., 2009). The skeleton of marine fishes on the other hand is composed of calcium phosphate rather than calcium carbonate as are otoliths. Nevertheless, it appears that the ossification of skeletal elements is also affected by pH stress. Crespel *et al.* (2017) found an increase in bone calcification and a lower occurrence of bone deformities in 45 days old sea bass larvae reared under elevated CO_2 levels (1520 ppm). This suggests that the observed faster skeleton development might be an indirect effect of internal

pH homeostasis and not caused by changes in the regulatory pathways. Nonetheless, the mechanism relating water CO_2 content to skeleton mineralisation remains unknown (Crespel et al., 2017). This highlights a major lack in our physiological understanding of ocean acidification effects in fish.

Previous data on increased larval mortality are important to interpret the ocean acidification effects on growth and development presented here (Stiasny et al., 2016). Some of the effects observed here may actually be a population-level rather than a physiological response, namely when particular phenotypic traits were correlated with susceptibility to ocean acidification, leaving, for example, more larvae in the population that showed increased growth under enhanced pCO_2. Unfavourable prey availability selects for phenotypes with increased growth (Folkvord, Rukan, Johannessen, & Moksness, 1997; Meekan & Fortier, 1996). It is well established that enhanced growth results in higher survival during early-life-stages, since larvae outgrow the predator field faster (Bailey & Houde, 1989). As another explanation, increased CO_2 concentrations may have led to increased foraging behaviour and food consumption (e.g. (*Amphiprion melanopus*) (Nowicki, Miller, & Munday, 2012) which may also apply to Atlantic cod larvae in our experiment and explain larger sizes under ocean acidification. Foraging behaviour or actual food consumption should be considered in future experiments.

A recent meta-analyses revealed that the data basis is still too limited to allow for generalizations with regard to ocean acidification effects on growth among fish species (Cattano *et al.*, 2018). The strong interaction between food supply and CO_2 treatment on growth and development observed in this study highlights that food regime is important in

assessing the impacts of ocean acidification on marine fishes. Therefore, some of the laboratory studies may reflect an incomplete or inaccurate picture of the likely responses especially for wild populations, since the majority are using ad libitum feeding conditions.

In conclusion it appears the cod larvae make the trade-off to spend more energy on growth and ossification of skeletal elements in the elevated CO_2 treatment compared to larvae in the ambient treatment, while putting less effort into organ development, when they are energy limited. While our study confirms increased larval size under ocean acidification - but only in an energy limited situation – it suggests metabolic trade-offs associated with near future CO_2 levels. This information is critically needed for the evaluation of ocean acidification effects. An increase in size may not translate into increased Darwinian fitness of larvae, i.e. enhanced reproductive contribution to the next generation. Increased larval size may result in developmental patterns that can possibly decrease fitness through decreased functionality of the organs or even impairments. To date, it remains unclear which factors drive these divergent responses and cause the larvae to invest so heavily into growth. Taken together, our study suggests that the responses of organisms and populations should be assessed as comprehensively as possible to not miss one of the critical biological effects.

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Figure Captions

Figure 1. Third ceratobranchial with gill filaments of 36 days old cod larvae showing (a) the measured lengths of the ossified gill arch (horizontal line), which is stained in pink, and the longest gill filament (vertical line) and (b) the measured gill filament area (shown encircled in red).

Figure 2. (a) Standard length of 36 days old cod larvae in mm and (b) Dry weight in mg (mean +/- SD) (N=11-26 per treatment).

Figure 3. Image of cleared and double stained specimens showing number of ossified vertebrae (coloured in red) in 36 days old cod larvae in (a) ambient CO2, high food (b) acidification, high food (c) ambient CO2, low food, (d) acidification, low food. (e) Number of fully ossified vertebrae in relation to standard length, lines show the linear relationship per treatment.

Figure 4. (a) RNA/DNA content of the 36 days old cod larvae (N=10-18 per treatment) and (b) Lipid content of the larvae (N=11-19 per treatment; mean +/- SD).

Figure 5. (a) Length of the ossified gill arch (mm) of 36 days old cod larvae (b) length of the longest gill filament (mm) (c) gill area (mm2) all in relation to standard length (in mm), lines show the linear relationships per treatment.

Figure 6. Frequency of organ impairments in (a) liver, (b) eyes, (c) pancreas, and (d) kidney of the 35 days old cod larvae in a subjective scale from 0 (no impairments) to +++ (very severe/many impairments). (N=6,5,3,9 (from left in each panel))

Author contributions

C.C., M.H.S. and M.S. designed the experiment; M.H.S., M.S., F.H.M. and C.C. performed the experiment; V.P. and A.M. supported the experiment and provided the facilities; N.K.S. performed the ossification analysis; I.B.F.P. performed the histological analysis; M.H.S., M.S., C.C. analyzed data; M.H.S., T.B.H.R., and C.C. wrote the main paper; All authors discussed the results and implications and commented on the manuscript at all stages.

Additional information

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