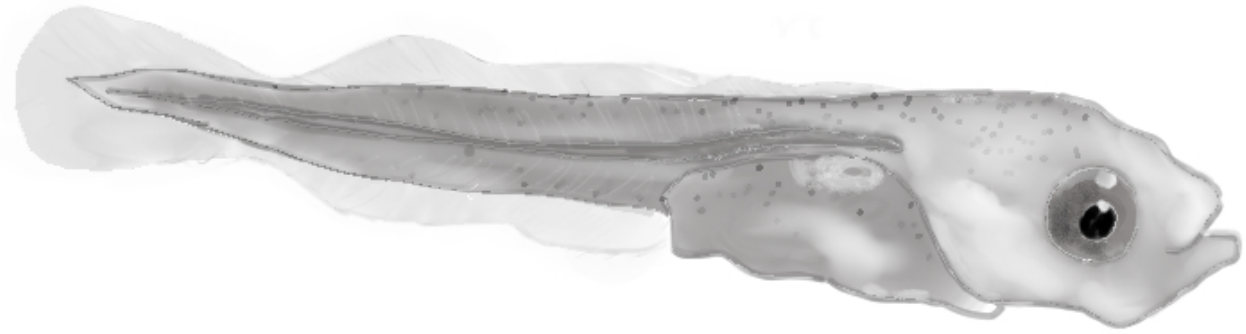

EFFECTS OF OCEAN ACIDIFICATION ON THE TRANSCRIPTOME OF LARVAL ATLANTIC COD AND IMPACTS OF PARENTAL ACCLIMATION

Dissertation zur Erlangung des Doktorgrades Dr. rer. Nat. der Mathematisch-Naturwissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel

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SUMMARY

The continuous combustion of fossil fuels and changed land use are resulting in increasing atmospheric concentrations of carbon dioxide (CO_2), one of the major greenhouse gases. This results not only in an increase of global temperature, but the direct dissolution of anthropogenic CO_2 into ocean waters causes "the otherCO_2 problem", ocean acidification. When CO_2 dissolves in oceanic water, it forms carbonic acid, which then partially dissociates into protons and bicarbonate ions leading to a decrease in seawater pH and a decrease in carbonate saturation state. Ocean acidification has been shown to impact many marine species, in particular calcifiers, while fish have been deemed resilient to this stressor. Recently, this picture has been revised as fish early life stages, i.e. eggs and larvae have been reported to being heavily impacted by increased CO_2 partial pressure (pCO_2). Most likely, the lack of gills during early developmental stages, the most important organ for acid-base regulation in adult fish, makes them susceptible to high pCO_2. My study species is the Atlantic cod (*Gadus morhua*), a keystone species in many parts of the North Atlantic with high commercial value. With a distribution from temperate to polar regions on both sides of the Atlantic we already observe pole-ward range shifts in this species, following the climate velocities to stay close to its thermal preferences. However, range shifts will not allow the escape from decreased ocean pH as changes, which to the contrary, are predicted to be most severe in the colder regions.

The objective of this dissertation was to understand how the early life stages of Atlantic cod are affected by simulated ocean acidification. For this, simulated levels of predicted ocean acidification in *in vivo* laboratory experiments were applied and various larval traits examined. First, the physiological phenotype under simulated ocean acidification is examined: growth, organ damages and bone development, considering the interaction with energy availability (Chapter 1). Then, whole transcriptome sequencing (RNA-Seq) is used to assess the underlying molecular phenotype and to elucidate the underlying transcriptomic mechanisms to the observed phenotypes (Chapter 2). Recent studies have presented evidence that transgenerational acclimation can result in reduction of the negative effects caused by ocean acidification. For Chapter 3, the objective was to assess the potential for mediation of the observed detrimental effects of increased pCO_2 caused by means of parental acclimation (5 months) by investigating various fitness related larval traits and gene expression.

Upon exposure to increased pCO_2, larval traits were affected differently depending on food availability. While *ad libitum* feeding caused increased growth compared to a restricted feeding regime regardless of the pCO_2 treatment, a general acceleration of development was observed. At a low, more realistic, feeding regime, larvae only grew larger under increased pCO_2, had more ossified bone structure, a better biochemical condition yet more frequent organ damage compared to control pCO_2. Generally, this observed increase in growth and ossification under simulated ocean acidification can be considered as an accelerated development similar to the development under high energy availability. This is further supported when examining gene expression patterns. Here, later larval developmental stages (36 days-post-hatch(dph)) showed 1413 differentially expressed genes between pCO_2 treatments. When

examining their annotated functions, it became evident that the observed changes in the transcriptome are most likely due to different developmental speed between the treatments rather than due to direct effects of ocean acidification. Even more interestingly, earlier larval stages (prior to gill development or with newly developed first gill filaments) showed very few changes in gene expression patterns, with only 3 and 16 genes differing, respectively. Particularly, the absence of differentially expressed genes related to acid-base regulation and the cellular stress response led to the conclusion that early cod larvae were either unable to perceive increased pCO_2 as a stressor, or were unable to respond to it transcriptomically. Thus, ocean acidification presents a "stealth stressor" to Atlantic cod larvae, considering the effects it has on mortality and organ damages despite the lack of a transcriptomic response.

In order to assess the effects of parental acclimation, parents were exposed to ambient and elevated pCO_2 conditions for five months prior to gamete collection. None of the previously mentioned effects of increased pCO_2 on the larvae were identified, independent of the parental acclimation. No changes in mortality, growth, respiration rate, ossification and other traits including whole transcriptome sequencing were observed. This is puzzling as numerous previous experiments have yielded such repeatable responses to simulated ocean acidification. But when examining the available information on the parental generation of fish in the experiment described in chapter 3, it became evident that a large part of the conducted research on the effects of ocean acidification on early life stages in fish – and possibly all other taxa - has a potential bias: When fish are under human husbandry, cultivation or breeding, most often their environment changes dramatically; lack of predators and competitors, controlled abiotic conditions and generally more resources potentially leading to a better condition are often observed. Favourable conditions could result in increased gamete quality which in case of many fish will lead to higher survival in the offspring. The five-month acclimation of the parental generation, including trice weekly *ad libitum* feeding, resulted in a much higher condition (doupling in hepatosomatic index) compared to the newly caught fish used in previous experiments (chapters 1 and 2), thus potentially leading to better provisioned eggs and more resilient larvae. A meta-analysis on all experiments addressing survival in cod larvae under increased pCO_2 clearly shows that survival is negatively impacted by simulated levels of ocean acidification. While this does not invalidate any previous finding of susceptibility or resilience of certain species to ocean acidification, it urges the need for control of the parents, the parental environments and conditions to properly assess the larval response and to create unbiased results applicable to wild populations. Due to the unexpected outcome of this experiment, the potential of long-term acclimation to predicted ocean acidification remains unanswered as well as the mechanisms causing the potential transgenerational acclimation.

Overall, this dissertation has provided several new insights into larval fish ecology under climate change. While larvae of Atlantic cod reacted phenotypically, by increased growth and bone ossification, to simulated levels of ocean acidification, no such patterns could be identified on the transcriptome level in direct response to increased pCO_2. Further, the experiment on the effects of parental acclimation and its results will stimulate the research on how not only parental acclimation but additionally parental condition impacts larval fitness under climate change.

ZUSAMMENFASSUNG

Die andauernde Verbrennung fossiler Brennstoffe und die veränderte Landnutzung führen zu steigenden Konzentrationen von Kohlendioxid (CO_2) in der Atmosphäre, einem der gefährlichsten Treibhausgase. Dies führt nicht nur zu einem Anstieg der globalen Temperatur, sondern die direkte Auflösung von anthropogenem CO_2 in Meerwasser verursacht auch „das andere CO_2-Problem": Ozeanversauerung. Wenn sich CO_2 in Ozeanwasser löst, bildet es Kohlensäure, die teilweise in Protonen und Bikarbonat-Ionen dissoziiert, was zu einer Abnahme des pH-Werts des Meerwassers und einer Abnahme des Sättigungszustands der Karbonatlösung führt. Ozeanversauerung beeinflusst viele Meeresorganismen, besonders jene, die Kalkstrukturen, wie z.B. Gehäuse und Schalen, bilden. Fische hingegen wurden als widerstandsfähig gegen diesen Stressfaktor eingestuft. Seit einiger Zeit wird diese Auffassung jedoch geändert, da frühe Lebensstadien von verschiedenen Fischarten, d.h. Eier und Larven, stark durch erhöhten CO_2-Partialdruck (pCO_2) beeinflusst werden. Die wahrscheinlichste Erklärung dafür ist das Fehlen von Kiemen in diesen Entwicklungsstadien, dem wichtigsten Organ für die Säure-Base-Regulation bei ausgewachsenen Fischen. Eine der Arten mit empfindlichen Larvenstadien ist der Atlantische Dorsch (*Gadus morhua*), eine Schlüsselart in vielen Teilen des Nordatlantiks und von hohem kommerziellem Wert. Da der Dorsch auf beiden Seiten des Atlantiks von gemäßigten bis in polare Regionen verbreitet ist, wird vermutet, dass er versuchen wird der globalen Erwärmung durch Pol gerichtete Migration zu entkommen. Jedoch stellt Migration keine Lösung dar, um einem verringerten pH-Wert des Ozeans zu entgehen, da die Veränderungen in den kälteren Regionen laut Vorhersage am gravierendsten sein werden.

Das Ziel dieser Dissertation ist es, das Wissen über den Einfluss von simulierter Ozeanversauerung auf die frühen Lebensstadien des Atlantischen Dorschs zu erweitern. Dazu wurden verschiedenste Merkmale in mehreren *in vivo* Laborexperimenten aufgenommen. Zunächst wurde der physiologische Phänotyp unter simulierter Ozeanversauerung untersucht: Wachstum, Organschäden und Knochenentwicklung, alles unter Berücksichtigung der Wechselwirkung mit Energieverfügbarkeit der Larven (Kapitel 1). Anschließend wurde Transkriptom-Sequenzierung (RNA-Seq) verwendet, um die molekularen Mechanismen aufzudecken, die den beobachteten Phänotypen zugrunde liegen (Kapitel 2). Jüngste Studien haben gezeigt, dass eine generationsübergreifende Akklimatisierung zu einer Verringerung der negativen Auswirkungen der Ozeanversauerung führen kann. Um eine mögliche Verminderung der schädlichen Auswirkungen von erhöhtem pCO_2 zu untersuchen, wurden verschiedene Fitnessmerkale an Larven untersucht, deren Elterngeneration zuvor 5 Monate bei verschiedenen pCO_2 Werten akklimatisiert wurde (Kapitel 3).

Wenn Dorschlarven einem erhöhten pCO_2 ausgesetzt waren, reagierten sie, abhängig von der Verfügbarkeit des Futters, in den untersuchten Merkmalen verschieden. Eine *ad libitum* Fütterung im Vergleich zu einem begrenzten Fütterungsangebot - unabhängig vom pCO_2 – bewirkte ein erhöhtes Wachstum und verursachte eine allgemeine Beschleunigung der Entwicklung. Bei dem niedrigen, realistischeren Fütterungsangebot waren die Larven nur unter erhöhtem pCO_2 größer, hatten eine stärker verknöcherte Wirbelsäule und eine bessere biochemische Kondition, jedoch häufigere

Organschäden im Vergleich zu der Kontrollgruppe. Im Allgemeinen kann dieser beobachtete Anstieg von Wachstum und Verknöcherung unter simulierter Ozeanversauerung als eine beschleunigte Entwicklung angesehen werden, ähnlich der Entwicklung unter hoher Energieverfügbarkeit. Dies wird weiter von den untersuchten Genexpressionsmustern unterstützt. Hier zeigten spätere Larvenstadien (36 Tage nach Schlupf) 1413 Gene, die differentiell zwischen verschiedenen pCO_2 Levels expressioniert wurden. Bei Betrachtung der annotierten Funktionen wurde es jedoch offensichtlich, dass die beobachteten Veränderungen im Transkriptom höchstwahrscheinlich auf die unterschiedlichen Entwicklungsgeschwindigkeiten, induziert durch die pCO_2 Behandlungen, zurückzuführen sind und nicht auf einen direkten Effekt der Ozeanversauerung. Noch interessanter ist, dass frühere Larvenstadien (vor der Kiemenentwicklung oder mit gerade entwickelten ersten Kiemenfilamenten) sehr wenige Veränderungen in den Genexpressionsmustern aufwiesen, wobei sich 3 bzw. 16 Gene unterschieden. Insbesondere das Fehlen von differentiell exprimierten Genen im Zusammenhang mit der Säure-Basen-Regulation und der zellulären Stressreaktion führte zu der Schlussfolgerung, dass frühe Dorschlarven entweder erhöhte pCO_2 Konzentrationen nicht als Stressor wahrnehmen können, oder nicht transkriptomisch darauf reagieren. Somit stellt die Ozeanversauerung einen *Stealth Stressor (getarnten Stressor)* für Atlantische Dorschlarven dar, in Anbetracht der Auswirkungen auf Mortalität und Organschäden bei fehlender transkriptomischer Antwort.

Um die Auswirkungen der parentalen Akklimatisierung zu beurteilen, wurde die Elterngeneration fünf Monate lang vor der Ei und Spermien Entnahme Kontroll- bzw. erhöhten pCO_2-Bedingungen ausgesetzt. Jedoch wurden keine der zuvor erwähnten Effekte, verursacht durch erhöhten pCO_2, auf die Larven, gefunden und dies unabhängig von der parentalen Akklimatisierung. Es wurden keine Veränderungen der Mortalität, des Wachstums, der Stoffwechselrate, der Verknöcherung und anderer Merkmale, einschließlich der Genexpression, beobachtet. Dies ist widersprüchlich, da zahlreiche frühere Experimente replizierbare Reaktionen auf simulierte Ozeanversauerung gezeigt haben. Bei der Untersuchung der verfügbaren Informationen über parentale Generationen von Fischen in verschiedenen Experimenten wird jedoch deutlich, dass ein großer Teil der durchgeführten Untersuchungen zu den Auswirkungen der Ozeanversauerung auf die frühen Lebensstadien von Fischen - und möglicherweise anderen Taxa - eine potenzielle Fehlerquelle aufweist: Wenn sich Fische in menschlicher Obhut befinden, ändert sich ihre Umwelt meist dramatisch; Fehlen von Räubern und Konkurrenten, kontrollierte abiotische Bedingungen und im Allgemeinen mehr Ressourcen, die möglicherweise zu einem besseren Allgemeinzustand führen können. Eine erhöhte Fitness könnte zu einer erhöhten Gametenqualität führen, die bei vielen Fischen in einer höheren Überlebensrate der Nachkommen resultiert. Die fünfmonatige Akklimatisierung der parentalen Generation, einschließlich der dreimal wöchentlichen *ad libitum* Fütterung, führte zu einer höheren Kondition im Vergleich zu der von neu gefangenen Fischen in früheren Experimenten (Kapitel 1 und 2), was potentiell zu qualitativ hochwertigeren Eiern und Larven führen könnte. Eine Meta-Analyse aller bisher durchgeführten Dorsch-Experimente zeigt deutlich, dass die Überlebensrate von Dorschlarven durch simulierte Ozeanversauerung negativ beeinflusst wird. Dies macht zwar keine früheren Befunde über Anfälligkeit oder Resilienz bestimmter Fischarten gegenüber Ozeanversauerung nichtig, legt jedoch nahe, die parentale Generation inklusive deren Umgebung und Fitness zu kontrollieren, um die Larvenreaktion richtig einzuschätzen und unvoreingenommene Vorhersagen für Wildpopulationen treffen zu können.

Aufgrund des unerwarteten Ergebnisses des Experiments, bleibt das Potential der langfristigen Akklimatisierung gegenüber der vorhergesagten Ozeanversauerung unbeantwortet, ebenso wie die Mechanismen, die die potentielle generationsübergreifende Akklimatisierung verursachen.

Insgesamt lieferte diese Dissertation wichtige neue Einblicke in die Fischlarvenökologie im Zeitalter des Klimawandels. Während Larven von Atlantischem Dorsch physiologisch, durch erhöhtes Wachstum und Verknöcherung auf simulierte Niveaus der Ozeanversauerung reagieren, konnten keine solche Muster auf Transkriptomniveau als direkte Reaktion auf erhöhten pCO_2 identifiziert werden. Darüber hinaus hat das Experiment durch die Erkenntnisse zur parentalen Akklimatisierung das Potenzial, die weiterführende Forschung insoweit zu stimulieren, nicht nur die parentale Akklimatisierung, sondern zusätzlich die parentale Kondition zu berücksichtigen, die die Larvenfitness unter Klimawandels beeinflussen kann.

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INTRODUCTION

WHAT IS OCEAN ACIDIFICATION?

The concentration of atmospheric carbon dioxide (CO_2) is constantly increasing since the onset of industrialisation, from 280 ppm (Caldeira & Wickett, 2003) to presently exceeding 406 ppm at Mauna Loa ((NOAA, 2018),1958 to present see figure 1). This increase is caused by combustion of fossil fuels, changed land use and other anthropogenic activities and results in the greenhouse effect, among other current changes summarized under "global change". As such, it is one of the characterising features of our epoch, the Anthropocene (Crutzen, 2002; Waters et al., 2016).

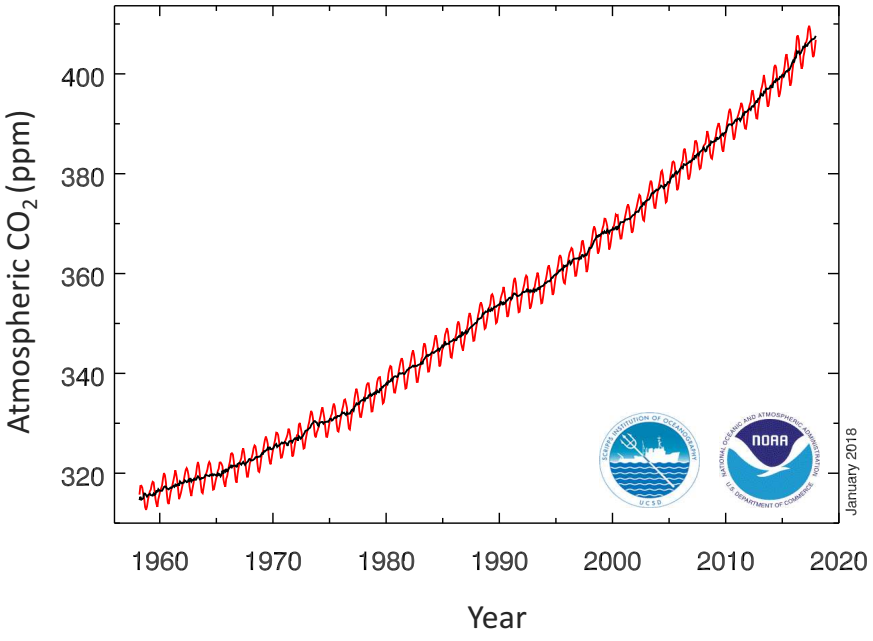

Figure 1: Increase of atmospheric carbon dioxide (CO_2) concentration since 1958 at Mauna Loa Observatory, Hawaii, United States. Adapted from NOAA.

An estimated third of the produced greenhouse gases are taken up by the ocean (Sabine & Feely, 2007); while this – in the long term – slows down further warming of the atmosphere, it does cause another problem: ocean acidification (OA), also been named "the other CO_2 problem" (Doney et al., 2009). When CO_2 is absorbed into the oceans, it reacts with water (H_2O) (formula 1) to form carbonic acid (H_2CO_3) which is found in equilibrium states with bicarbonate (HCO_3^-) and free hydrogen ions (H^+) and further, carbonate (CO_3^{2-}) with free hydrogen ions (H^+).

$$\textbf{(1)}\quad CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

The increase in protons is measured as a decrease in ocean pH and results in changes in the ocean carbonate chemistry. At pH 8.1, 90 % of all inorganic carbon is found in form of bicarbonate. This percentage increases under ocean acidification while the amount of carbonate decreases, lowering the calcium carbonate saturation state (Orr et al., 2005; Doney et al., 2009). This effect has already been observed as a decrease of ocean surface water pH from 8.2 to 8.1 since pre-industrial times. It is predicted to decrease further down to a mean global sea surface pH of below 7.8 or 7.9 by the year 2100, depending on different emission scenarios (RCP8.5 and RCP4.5, respectively), this would correspond to the atmospheric CO_2 concentrations (IPCC, 2013) of ~ 960 µtam or 550 µtam (see figure 2). Even though variations in atmospheric CO_2 levels have occurred throughout earth history, the current rate of change is unprecedented over geological time (Hönisch et al., 2012). Climate change is already impacting the marine fauna (Poloczanska et al., 2013). While ocean warming can be evaded by poleward migration and changes in distribution ranges, as already observed in fish populations (Perry et al., 2005), no such migrations provide refugia from predicted increases in ocean pCO_2 exposure to ocean acidification.

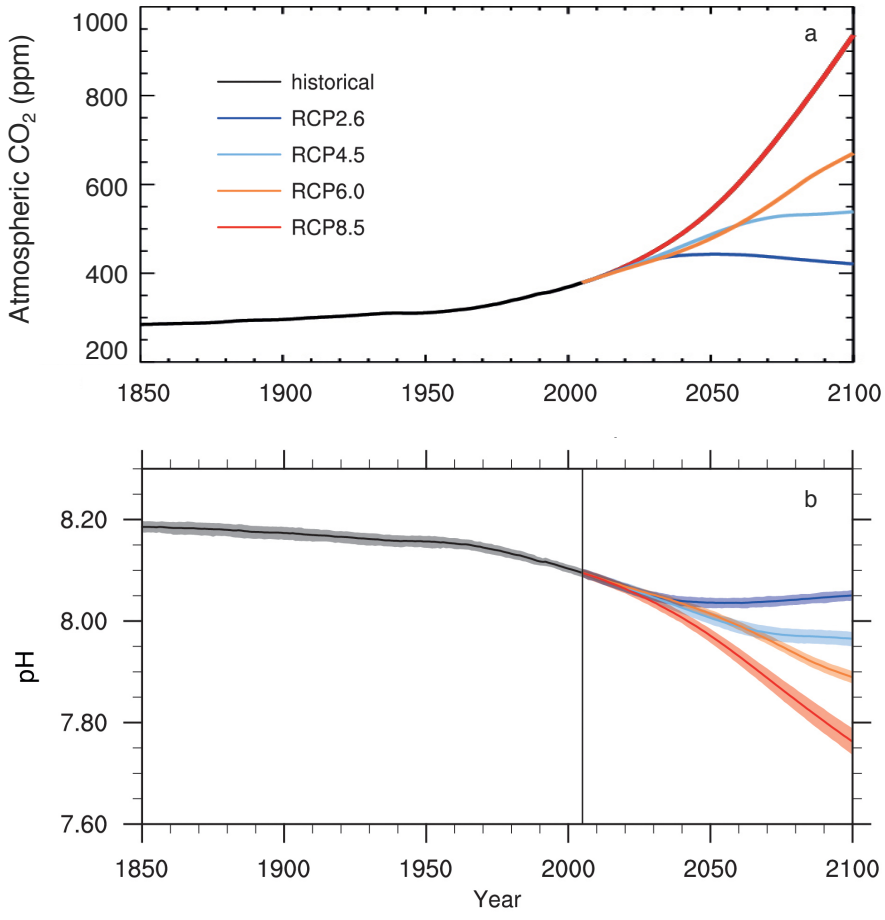

Figure 2: Predicted changes in atmospheric CO_2 concentrations (ppm) (a) and global mean ocean surface pH according to different representative concentration pathways (RCP) until year 2100. Adapted from Stocker et al. (2013).

IMPACTS OF CHANGED OCEAN pCO_2 ON MARINE FAUNA

Simulated ocean acidification by experimentally increasing pCO_2 levels has been shown to affect many marine organisms (Brierley & Kingsford, 2009; Kroeker et al., 2013). Particularly, calcifying species are threatened by increased pCO_2 including habitat forming species such as corals (Hoegh-Guldberg et al., 2007; Anthony et al., 2008) but also molluscs (Gazeau et al., 2013) and calcifying plankton such as coccolithophores (Zondervan, 2007; Schlüter et al., 2014, Riebesell et al., 2000). These detrimental effects are most likely due to decreases in ocean pH (Thomsen et al., 2015) and/or reduced carbonate saturation states in surface waters (Orr et al., 2005; Waldbusser et al., 2015). These changes could lead to a reduction of biodiversity and productivity through habitat loss, e.g. lost coral reefs (Pandolfi et al., 2011; Poloczanska et al., 2013; Hoegh-Guldberg et al., 2014; Nagelkerken et al., 2016). Unlike many vertebrates, fish are believed to be resilient to the effects of predicted ocean acidification (reviewed by Ishimatsu et al. (2008)). Indeed, adult fish are effective osmoregulators allowing them to cope with very high levels (above 3000 µatm to 15000 µatm) of dissolved CO_2 under extended periods of time without increased mortality or changes in growth (Foss et al., 2003) or swimming and metabolic performances (Melzner et al., 2009b). This is mainly due to fish gills being a very efficient organ for ion exchange and thus, acid base regulation because of the high concentration of specialized epithelial cells (mitochondrial rich cells) (Claiborne et al., 2002; Gilmour & Perry, 2009). As gills are only formed during development from newly hatch larvae to juvenile, the lack of these in early life stages would explain the detrimental effects simulated ocean acidification has on the larvae of some fishes.

EFFECTS OF OCEAN ACIDIFICATION ON EARLY LIFE STAGES OF FISH

Early life stages of fish have often not been included when summarizing fish susceptibility to ocean acidification. But lately the number of studies addressing the effects of many different pCO_2 levels simulating ocean acidification on early life stage of a variety of fishes has increased. The impact of ocean acidification on early life stages is equally as important as the effects on the adults. Further, larvae often present the bottleneck for recruitment and population growth (Houde, 2008; Llopiz et al., 2014). Even if adult fish are hypothetically entirely unaffected by predicted levels of ocean acidification, if a single early life stages is highly susceptible, reproduction would not be successful, resulting in population decline. Thus, knowledge on the effects on all life stages is important. The following chapter will summarize the current state of knowledge on early life stages, the most vulnerable stages, in reaction to ocean acidification. As this dissertation is focusing on the impacts of simulated ocean acidification of larval Atlantic cod (see box) special emphasis is put on the effects on this species.

Various levels of increased pCO_2 (1260-4635 µatm) did not result in changes in embryonic development and hatching success in herring compared to ambient conditions (Franke & Clemmesen, 2011). While similar patterns of embryonic development were since observed for other species (Hurst et al., 2013; Tseng et al., 2013). Other studies identified species that suffered decreased hatching success under near future predicted ocean acidification levels (Chambers et al., 2014; Faria et al., 2017) and in one case, a decrease in embryonic survival (Forsgren et al., 2013). Time to hatch was also impacted in one study (Hurst et al., 2013) while others showed no effect (Munday et al., 2009a; Frommel et al., 2013; DePasquale et al., 2015). While cod eggs from the Eastern Baltic stock were not affected in relation to

egg survival and hatching success under increased pCO_2 (Frommel et al., 2013), eggs of the North East Atlantic migratory cod (NEA) were impacted showing decreased hatching success, smaller size at hatch and increased embryonic oxygen consumption (Dahlke et al., 2017).

Decreased survival of early life stage caused by increased pCO_2 has been reported for several fish species (Baumann et al., 2012; Miller et al., 2012; Murray et al., 2014; Bromhead et al., 2015; Frommel et al., 2016) while equally many and diverse studies found no effect (Hurst et al., 2013; Chambers et al., 2014; DePasquale et al., 2015; Mu et al., 2015; Munday et al., 2016). Interestingly, some studies have even found a positive effect of increased pCO_2 on the survival of larvae (Pope et al., 2014; Diaz-Gil et al., 2015). Even susceptibility within species has been shown to vary. While Eastern Baltic cod larval survival was not effected by very high pCO_2 levels (1260-4000 μatm) (Frommel et al., 2013), both Western Baltic Sea and Barents Sea cod showed a dramatic increase in larval mortality when exposed to 1100 μatm (Stiasny et al., 2016). So far, no definitive mechanism has been identified that causes the changes in survival under different levels of ocean acidification; particularly, no mechanism that would explain the various affects in different species and populations. A large part of communicated susceptibility of larval fish to ocean acidification is based on observed changes in behavioural patterns due to increased pCO_2 (Munday et al., 2009b; Dixson et al., 2012; Nilsson et al., 2012; Welch et al., 2014) and have been suggested to be caused by disrupting gamma-aminobutyric acid (GABA) recepter function by changes in ion concentration in the extracellular fluids (Nilsson et al., 2012; Regan et al., 2016). This body of research is however currently highly discussed as problems with reproducibility of the results have questioned their universal applicability (Sundin et al., 2017). For Atlantic cod larvae, swimming kinetics and behaviour remained generally unaffected (Maneja et al., 2013b).

Other often examined traits in larval fishes are related to oxygen consumption rate and linked to energy metabolism. The proposed mechanism is an increase in seawater pCO_2 causing an increased energy demand due to the increased Na^+/K^+ ATPase activity to retain the Na^+ gradient between gill and seawater allowing for proton export (Gilmour & Perry, 2009). But while *Amphiprion melanopus* (Miller et al., 2012) under 1032 μatm showed an increase in oxygen consumption, Pimentel et al. (2014a, 2014b) reported decreased metabolic rates for *Coryphaena hippurus* and *Solea senegalensis*. However, none of the studies were able to conclusively address the causes of changes in oxygen consumption in relation to increased environmental pCO_2. The hypothesis that increased energy demand, due to ion regulation cost, would result in smaller larvae as less energy remains for growth and development (Melzner et al., 2009a) is not universally confirmed in fish larvae. Both decreases and increases in body size have been observed (compare Baumann et al., 2012; Pimentel et al., 2014a, 2016; Frommel et al., 2016 to Chambers et al., 2014; Pimentel et al., 2016). Early (non-feeding) larval stages of Eastern Baltic cod did not show any changes in growth (Frommel et al., 2013) while larvae from a Norwegian coastal cod population were heavier under increased pCO_2 at 32 dph (Frommel et al., 2012). As for survival, the entirety of underlying mechanisms of changes in growth patterns remains elusive.

One of the most severe results of simulated ocean acidification on fish larvae is found as tissue damages to several organs as reported by Frommel et al. (2014, 2016) and Chambers et al. (2014) for yellowfin tuna, Atlantic herring and summer flounder. A number of studies (Baumann et al., 2012; Forsgren et al., 2013; Chambers et al., 2014; Pimentel et al., 2014a, 2016; Ahnelt et al., 2015; Mu et al., 2015) also found

skeletal or developmental malformation in response to simulated levels of ocean acidification. Atlantic cod larvae reared in 1800 and 4200 μtam pCO_2 showed severe tissue damages in several organs including eyes, kidney and liver (Frommel et al., 2012).

Measurements related to otoliths (ear stones of fishes) in fish larvae vary and cannot easily be interpreted but otolith size is generally increasing under simulated ocean acidification (Checkley et al., 2009; Munday et al., 2011a; Bignami et al., 2013; Pimentel et al., 2014a). However, Franke et al. (2011) and Munday et al. (2011b) found no changes. In Atlantic cod, Maneja et al. (2013a) reported on increase in otolith area under increased pCO_2. Interestingly, bone ossification, at least in early juvenile sea bass, seems to be accelerated under simulated ocean acidification (Crespel et al., 2017). The increase of calcification (deposition of $CaCO_3$) of otoliths could be explained by increased HCO_3^- concentrations and thus substrate availability for calcification in the endolymph surrounding the otolith (Checkley et al., 2009), but no explanations are so far given for changes in bone ossifications as these are mainly consisting of calcium phosphate (hydroxyapatite) deposits versus calcium carbonate in the otoliths.

Other, more rarely examined traits include biochemical indicators (RNA/DNA) reflecting on the state of the protein synthesis capacity of larvae (Franke & Clemmesen, 2011; Frommel et al., 2014), energy metabolism (Silva et al., 2016), heart rate of embryos and larvae (Mu et al., 2015; Lonthair et al., 2017) as well as lipid content (Diaz-Gil et al., 2015) and composition (Murray et al., 2017), and feeding rate (Pope et al., 2014). RNA/DNA ratios were not affected in Eastern Baltic cod (Frommel et al., 2013) neither were they affected by any of the applied pCO_2 levels in Norwegian coastal cod (Frommel et al., 2012). Additionally, 32 day old larvae of Norwegian coastal cod had a significantly higher content of lipids under high pCO_2 compared to ambient.

In comparison to adult fish, very little on the acid base regulatory mechanisms and abilities of early life stages is known (Melzner et al., 2009a) and thus information on the causes and mechanisms underlying the susceptibility to ocean acidification is limited for eggs and larvae, compared to adult fish. As eggs and early larvae lack gills, ion exchange is generally limited to so called chloride cells before functional gills are developed. These Na^+/K^+ ATPase-rich ionocytes are found on the epithelia and yolk sacks of larvae (Falk-Petersen, 2005; Dahlke et al., 2017).

ACID-BASE REGULATION IN RESPONSE TO OCEAN ACIDIFICATION IN FISHES

The regulation of extracellular fluid pH, such as blood and haemolymph, has been suggested to be a crucial characteristic of species tolerant to high pCO_2 as a lack of the same could lead to metabolic depression (Melzner et al., 2009a and references within) and cause a change from aerobic to anaerobic metabolism (Michaelidis et al., 2007). The main buffering system in blood/haemolymph responding to decreases in pH is the CO_2-carbonate system including the interaction of pH, pCO_2 and bicarbonate (HCO_3^-). To summarize, an increase in pCO_2 causes a decrease in extracellular pH, which, in case of acid base regulators, is compensated over time through buffering with increased HCO_3^-. This returns the extracellular pH back to previous levels (for visualization see Fig 1 in Davenport, 1974). Carbonic anhydrase (CA) is the key catalyst in this context, hydrating CO_2 into HCO_3^- and protons in most tissues (fish are lacking functional CA in the plasma (Melzner et al., 2009a), and is concentrated in the gills and

red blood cells. In the gill's epithelial cells, protons are exported into the surrounding seawater through H^+/Na^+ exchangers. In the majority of studied fish species, pH returned to levels prior to acidosis within the time frame of hours to days (reviewed in Heuer & Grosell, 2014). However, although pH is back to original values, the extracellular pCO_2 and HCO_3^- concentrations remain elevated. In addition to the described bicarbonate buffering, protons can be bound in the non-bicarbonate buffering system, mainly constituting of amino acids and organic phosphate groups (Melzner et al., 2009a). Further, CO_2 can be exported directly in a gaseous form. To allow for a constant net export of CO_2 from the extracellular fluids into the surrounding seawater, a pressure gradient is achieved by extracellular pCO_2 levels far exceeding environmental levels (generally 2600 to 4900 µatm) (Perry & Gilmour, 2006; Melzner et al., 2009a). Further, the above-mentioned export of protons via H^+/Na^+ exchangers requires an Na^+ gradient between cell and surrounding seawater, which is achieved by the energy dependent Na^+/K^+ ATPase.

Complementary to the extracellular pH, the intracellular pH is highly important in many catalytic functions and processes and needs to be tightly controlled in comparison to extracellular pH. This is primarily achieved through Cl^-/HCO_3^- and H^+/Na^+ exchange (Parks et al., 2010). Deficiencies in pH regulation can result in a severe decrease of extracellular pH, causing acidosis, which in turn can affect metabolic pathways (Heisler, 1989).

Few studies to date have assessed gene expression patterns in larval fish in response to increased pCO_2. The few available studies have focused on genes involved in acid-base regulation. To the best of our knowledge, only one study has investigated candidate gene expression patterns in the larval stage (Tseng et al., 2013) and an additional study has focused on the early juvenile stages (Crespel et al., 2017). While Tseng et al. (2013) identified several differences in transcript abundance in larval medaka (*Oryzias latipes*), e.g. differential expression of NHE3 (SLC9a1), CA2 and AE1a (SCL4a1) genes, Crespel et al. (2017) found no changes in gene expression patterns using both candidate genes and micro arrays in sea bass juveniles (*Dicentrarchus labrax*). But until now, no study has performed whole transcriptome analysis of larval fish exposed to simulated ocean acidification yet it could provide valuable information, unbiased by candidage gene selection, on how larval fish react to increased pCO_2 on the gene expression level. Allowing us to potentially understand the underlying molecular mechanisms to observed phenotypic reactions.

Study organism: Atlantic Cod

Atlantic cod (*Gadus morhua,* L.) is one of the commercially and ecologically most important fish species in the North Atlantic. It is a demersal fish found along the majority of the American and European coastline as well as in a large portion of the continental shelfs and the Baltic Sea, inhabiting temperate to arctic climate zones.

Atlantic cod is valued for its taste and is fished recreationally and commercially, using various methods such as trawls, gill nets, traps and hook-and-line. Depending on the population cod perform spawning migrations of several hundred kilometres where they aggregate in spawning areas where they spawn a large, depending on female size, number of relatively small eggs. These eggs drift in the water column until they hatch. The early larvae, upon depletion of their yolk sac start to feed on zooplankton. After metamorphosis from larvae to juvenile, cod start to feed on an omnivorous diet that can shift with season, location and size. Atlantic cod is listed as "vulnerable" in the IUCN Red List (IUCN, 2017).

Its habitats, the Arctic Atlantic Ocean including the Barents Sea, home of the largest cod population, the Northeast Arctic Cod (NEAC), in particularly are threatened by warming as well as ocean acidification, the latter due to its increased uptake of CO_2 into cooler waters as well as its inherent low carbonate concentration (low C_T to A_T) (AMAP, 2013). The Barents Sea and the cod populations there are predicted to experience the pCO_2 and pH levels globally predicted for the end of the century, as early as the mid-21st century (Skogen et al., 2014).

Atlantic cod was chosen as an object for this investigation due to its ecological and economical importance as well as to the previously suggested susceptibility of larval stages to the effects of increased pCO_2 (Frommel et al., 2012; Stiasny et al., 2016).

EFFECTS OF OCEAN ACIDIFICATION ON ATLANTIC COD

In addition to the investigations on early life stages of cod (Frommel et al., 2012, 2013, Maneja et al., 2013a, 2013b; Stiasny et al., 2016; Dahlke et al., 2017) (see section "Effects of ocean acidification on early life stages of fish"), numerous other studies have examined the effects of increased pCO_2 conditions on juveniles and adults of Atlantic cod covering an even wider range of investigated parameters and variable exposure durations (Melzner et al., 2009b; Moran & Støttrup, 2011; Moran et al., 2012; Jutfelt & Hedgärde, 2013, 2015, Kreiss et al., 2015a, 2015b; Stapp et al., 2015; Schmidt et al., 2017a, 2017b). Further, two studies have so far investigated the changes in gene expression patterns in response to increase pCO_2 of selected candidate genes related to acid-base regulation in my study organism, Atlantic cod (Hu et al., 2016; Michael et al., 2016). While Michael et al. (2016) focused on genes related to ion transport and pH regulation in the gills, Hu et al. (2016) investigated the effects of increased pCO_2 and temperature on the intestinal ion transport, both of juvenile and adult cod. In gills, expression of several ion transporters, such as NHE1B and Na^+/K^+-ATPase α subunits were upregulated in response to increased pCO_2 (1100 μatm) under thermal optimum (10 °C) compared to ambient pCO_2 treatments, while the HCO_3- transporter (SLC26A6) was downregulated. These patterns however, were very different at warm (18 °C) and cool temperatures (10 °C) under very high pCO_2 levels (2200 μatm). Interestingly, protein levels of ion transporters did not vary under different pCO_2 treatments at optimal temperatures, but were increased at higher temperatures combined with medium pCO_2 levels compared to ambient and high CO_2 treatments(Michael et al., 2016). In the anterior intestine of the same cod, only

differential gene expression for Na^+/K^+-ATPase ATP1a1, Na^+/H^+ exchanger NHE3, Na^+/HCO_3^- exchanger NBCa, HCO_3^- transporter SLC26A6 and carbonic anhydrase CA15a was identified between ambient and high CO_2 treatments, while no change was observed between ambient and medium treatments under 10 °C (Hu et al., 2016). On the other hand, fish acclimated to 18 °C only showed differences in gene expression in HCO_3^- transporter SLC26A3.2 and most prominently, a drastic decrease of carbonic anhydrase (CA2) mRNA abundance.

With the exception of two publications (Frommel et al., 2013; Maneja et al., 2013b), all larval studies show a strong effect of simulated ocean acidification an larvae of Atlantic cod, no conclusive impacts of ocean acidification on juvenile and adult cod and other *Gadidae* could be found. While numerous of these studies identified effects, often applying very high pCO_2 levels in additional combination with high temperature treatments; numerous other investigated traits were not affected at all. In view of all these findings, juvenile and adult cod can be considered relatively unaffected on a phenotypic level to predicted levels of pCO_2, even though changes in gene expression suggest other metabolic and energetic ramification and potential side effects that need further investigation. Under the current state-of-knowledge, particularly considering the effects that simulated ocean acidification has on larvae; Atlantic cod as species has to be considered highly impacted by predicted ocean acidification.

ADAPTATION, ACCLIMATION AND TRANSGENERATIONAL PLASTICITY

Generally, most studies investigating the effects of ocean acidification on marine organisms have focused on a single life stage with relatively short exposure times, even though the predicted increase in ocean pCO_2 is not an immediate change, but a continuous rise. Further, assessment of one life stage does not allow an evaluation of a species' overall vulnerability to the stressor, if one life stage is highly impacted, the organism should be considered susceptible to the increased pCO_2. Another issue with single generation approaches are the direct exposure experiments and their exclusive consideration of the immediate effect of predicted pCO_2 levels on the present-day individuals. They do not take the possible mediation of effects due to transgenerational acclimation or even adaptation into account (Kelly & Hofmann, 2013; Crozier & Hutchings, 2014; Sunday et al., 2014).

When organisms are experiencing changes in their environment, it will most likely result in a change of phenotype over time (reviewed by Merilä & Hendry, 2014). These changes can either be caused by evolutionary adaptations on a population scale or by phenotypic plasticity (Reusch, 2014). While it has been suggested that most populations could accommodate a standing genetic variation which would allow for evolutionary adaptation to the effects of climate change by positive selection of certain genotypes of higher fitness (Shaw & Etterson, 2012; Pespeni et al., 2013). The majority of experimental evidence for evolutionary adaptation in response to climate change is found for organismes with short generation times, such as marine pytoplankton (Lohbeck et al., 2013; Hoffmann & Sgró, 2011).

Phenotypic plasticity, the change of a phenotype in response to the environment without the change of the underlying genotype, or the ability of one genotype to produce several phenotypes (West-Eberhard, 1989) is the other way to change an organism's phenotype in response to environmental change. Plasticity can be separated into three categories: reversible, developmental and transgenerational

plasticity (Donelson et al., 2017). Reversible and developmental plasticity are defined to one generation. In the case of reversible plasticity, once the stressor has declined the phenotype changes back to its original state while changes due to developmental plasticity are irreversible changes to the phenotype. If the observed phenotype, however, is the result of the interaction between the environment experienced by the present and the previous generation, the plasticity is transgenerational (Salinas et al., 2013; Donelson et al., 2017). The effects of transgenerational plasticity (TGP) have been described under various synonyms such as maternal effects (Burgess & Marshall, 2011) , transgenerational acclimation (Donelson et al., 2012) and anticipatory parental effects (Uller et al., 2013). TGP is generally considered to be regulated by epigenetic modifications that alter the phenotype of the observed generation in relation to the previous generations (Jablonka & Raz, 2009).

Studies that have addressed the potential of TGP in response to ocean acidification, show at least partial mediation (as a reduction of the negative effects) in response to the stressor (Thor & Dupont, 2015), while other studies could only identify such mediation by transgenerational acclimation in some of the examined traits (Parker et al., 2012; Chakravarti et al., 2016). In the reef fish, *Acantochromis plyacanthus,* parental exposure to increased pCO_2 did not lead to any improvement of lateralizing behaviour or response to olfactory cues, both heavily impacted under simulated ocean acidification (Welch et al., 2014). *Amphiprion melanops* on the other hand showed general improvement in affected traits under increased pCO_2, namely survival, length, weight, metabolic rate (Miller et al., 2012) and behaviour (Allan et al., 2014) when the parental generation was exposed to the same pCO_2. *In situ,* seasonal variation in coastal pH experienced by the parental *Menida menida* generation appears to affect the resilience of larvae from different seasons in response to increased pCO_2 (Murray et al., 2014). Various studies applying other stressors than increased pCO_2, found positive, neutral and/or negative effects of transgenerational exposure under many conditions (Shama & Wegner, 2014; Donelson et al., 2016; Shama et al., 2016; Le Roy et al., 2017). Overall, the impact of parental acclimation on the offspring fitness has been shown to strongly dependent on the duration of the parental exposure time (Miller et al., 2012; Salinas & Munch, 2012; Suckling et al., 2015). This could be explained by the start of the parental exposure in relation to the duration gametogenesis (Dupont et al., 2013) or by environemtal stability, as a stabil environment is more likely to result in TGP (Donelson et al., 2017).

Parental exposure to a stressor, however, is not always beneficial for the offspring. Only 42 % of the transgenerational studies found a positive effect of parental exposure to the stressor, but in 22 % of the examined cases negative, accumulative effects were shown (Donelson et al., 2017). An earlier review concluded that positive effects of transgenerational acclimation are rare and evidence in most cases is weak (Uller et al., 2013).

To summarize, in some of the cases, TGP caused by parental exposure or acclimation in addition to offspring exposure, has the potential to reduce the adverse effects of ocean acidification on fish larvae compared to the direct effects of ocean acidification without previous acclimation of earlier generations. It is however not universally applicable and where applicable, TGP rarely fully mediates the effects e.g. restoring the phenotype to “unstressed” levels. Due to the long generation time, often large body size and difficult larval rearing techniques, no studies to date have so far examined whether this is also applicable to commercially important, non-model fish species.

METHODS AND OBJECTIVES OF THIS DISSERTATION

Whole transcriptome analysis, e.g. analysis of gene expression patterns, is a well-established tool to assess the connection between the interaction of genotype and environment to the phenotype (DeBiasse & Kelly, 2016). In order to be able to apply and transfer the knowledge of experimental results to other species, not yet experimentally examined, a deeper understanding of the underlying physiological mechanisms of the observed responses and phenotypes caused by simulated ocean acidification is needed. Here, particularly the acid-base regulatory mechanism and the differences in regulatory abilities between vulnerable and resilient species are of interest and need to be addressed. If particular genes or regulatory pathways are differentially expressed, they can be identified by whole transcriptome sequencing, under ocean acidification, and follow-up hypotheses of what could potentially be causing the observed effects can be formulated and addressed. No study has so far investigated the effect of increased pCO_2 on the global transcriptome of larval fish using RNA-Seq (See Oomen & Hutchings, 2017 for recent review). Further, only very few studies have implemented this method on adult or juvenile fishes to assess certain aspects of their response to ocean acidification (Schunter et al., 2016; Crespel et al., 2017; Hamilton et al., 2017)

This dissertation has, besides the overall aim to increase understanding of larval responses to simulated ocean acidification, four research objectives:

Objective 1: How are larvae of Northeast Arctic cod impacted by direct exposure to predicted levels of ocean acidification on a phenotypic level (growth, bone ossification, tissue damages and other traits)?

Objective 2: How are these phenotypic responses (identified by objective 1) correlated with differential gene expression (RNA-Seq) in larvae during development?

Objective 3: Does parental acclimation (5 months) to high pCO_2 vs. ambient levels cause changes to the phenotypic response (identified by objective 1) of larvae exposed in full combination with parental acclimation to elevated pCO_2?

Objective 4: How do the changes in phenotypic response due to parental acclimation to elevated pCO_2 (identified by objective 3) correlate to differential gene expression of larvae exposed to simulated ocean acidification?

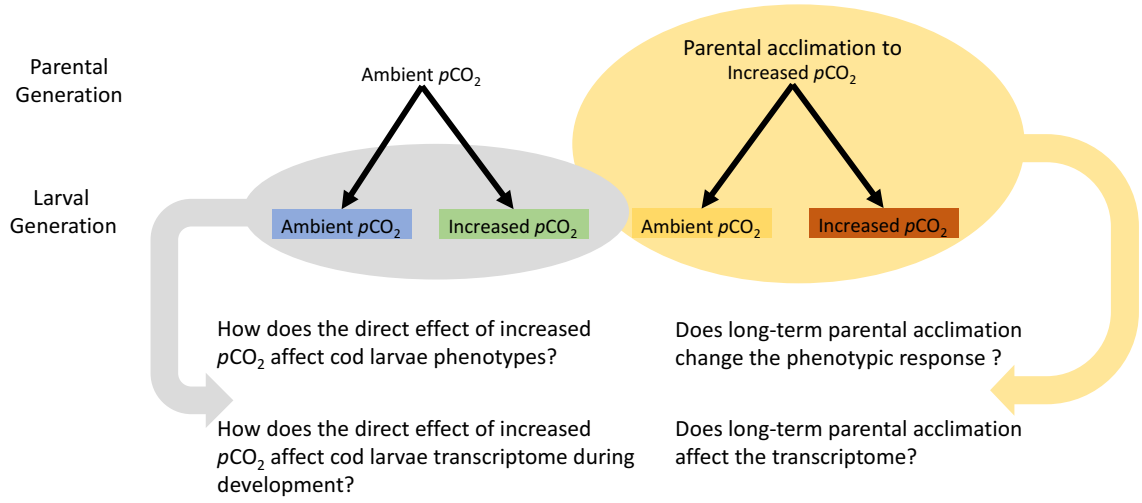

Figure 3: Schematic summary of objectives and related questions addressed in this dissertation

DISSERTATION OUTLINE

This dissertation comprises of three chapters, each representing a manuscript (submitted or to be submitted) to a peer-reviewed journal. Each of the following three chapters addresses one or several of the above-mentioned objectives. A statement containing the contributions of all authors is found individually in each manuscript as well as in the Appendix. Bibliographies for each chapter are placed at the end of the corresponding manuscript while supplementary information is to be found in the appendix. A bibliography containing references of the Introduction as well as Discussion and Synthesis are found after the Concluding Remarks, while references in the chapters are to be found in the respective bibliography of each chapter.

Chapter 1.

Impacts and trade-offs of ocean acidification on growth, skeletal, and organ development of Atlantic cod larvae

M. Stiasny, M. Sswat, F. Mittermayer, I-B Falk-Petersen, N. Schnell, V. Puvanendran, A. Mortensen, TBH Reusch and C. Clemmesen

This chapter addresses the question how larval cod are phenotypically affected by predicted end-of-century pCO_2 levels (objective 1)? The phenotypic response of Atlantic cod larvae to predicted end-of-century levels was assessed in regard to growth, bone ossification, gill development and histological analyses of organs/tissue. Further, the energy availability, e.g. prey density offered to the larvae as a potentially buffering effect was analysed.

This Chapter has been submitted to Global Change Biology.

Chapter 2.

Transcriptome profiling reveals exposure to predicted end-of-century ocean acidification is a stealth stressor for Atlantic cod larvae

F. Mittermayer, M. Stiasny, C. Clemmesen, S. Jentoft, T. Bayer, V. Puvanendran, M. Chierici und T.B.H. Reusch

This chapter looks at how different larval stages react on the gene expression level to predicted end-of-century pCO_2 levels (objective 2). The global gene expression patterns of larvae at three different developmental stages (6, 13 and 36 days post hatch) under predicted end-of-century pCO_2 were investigated, applying global mRNA sequencing and quantification (RNAseq). Further emphasis was put on genes involved in acid-base regulation, the cellular stress response and genes that could potentially be related to changes in the phenotypes observed in Chapter 1.

This chapter has been submitted to Scientific Reports.

Chapter 3.

Husbandry effects in experiments addressing ocean acidification: interaction with direct exposure and long-term parental acclimation to increased pCO_2 in Atlantic cod

F. Mittermayer, M. Stiasny, G. Göttler, N. Schnell, S. Jentoft, J. Barth, M. Matschiner, P. Berg V. Puvenandran, A. Mortensen, P. Thor, C. Bridges, M. Chierici, T.B.H. Reusch, C. Clemmesen

This chapter addresses the question whether or not parental acclimation to relevant pCO_2 levels allow for a higher fitness in phenotypic response (objective 3) or whole transcriptome (objective 4) of larvae. For this study, the parental generation was incubated either at ambient or at elevated pCO_2 levels for five months prior to gamete collection. Eggs and larvae were subsequently either reared in the same or the opposite of the parental treatments. Phenotypic responses of larvae such as mortality, growth, respiration, biogeochemical condition, bone ossification and development as well as transcriptome analysis were performed to assess the impact of parental acclimation on the susceptibility of Atlantic cod larvae to predicted end-of-century pCO_2 levels.

This chapter is awaiting submission after further revision with the co-authors.

CHAPTER 1

IMPACTS AND TRADE-OFFS OF OCEAN ACIDIFICATION ON GROWTH, SKELETAL, AND ORGAN DEVELOPMENT OF ATLANTIC COD LARVAE

Impacts and trade-offs of ocean acidification on growth, skeletal, and organ development of Atlantic cod larvae

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Abstract

In order to understand the effect of global change on marine fish populations, 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 show that larval growth among 35-36 days old surviving larvae is also affected, along with organ development and ossification of the skeleton. We combined CO_2-treatments (ambient: 503 ppm, high: 1179 ppm) with food availability in order to evaluate the effect of energy limitation in addition to the ocean acidification stressor. Larval size and skeletogenesis were positively affected by high food availability. We found significant interactions between acidification and food availability in that larvae fed *ad libitum* showed little difference in growth and skeletogenesis between the ambient and high CO_2 treatment. In contrast, larvae under energy limitation were significantly larger and had further developed skeletal structures in the acidified treatment compared to the ambient CO_2 treatment. However, the latter group revealed impairments in certain 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. For the first time, our study highlights important allocation trade-off, which the larvae have to make between growth and critical developmental processes.

Introduction

Over the last two centuries anthropogenic activities have led to an increase in atmospheric concentrations of carbon dioxide, which are predicted to reach levels of about 750-1000 ppm CO_2 by the end of the century depending on the different emission scenarios used (IPCC, 2014). Significant effects on marine ecosystems are to be expected (Orr *et al.*, 2005; Doney *et al.*, 2009) through changes in ocean acidification levels and increases in temperature via the indirect effects of CO_2 as a greenhouse gas. Commercially and ecologically important fish populations are progressively subjected to these global changes, for example through increasing temperatures leading to a northward movement of fish stocks (Perry *et al.*, 2005; Poloczanska *et al.*, 2013) and due to the higher solubility of CO_2 in colder waters leading to an increase in ocean acidification levels (Steinacher *et al.*, 2008). A thorough understanding of these effects on individuals and populations becomes increasingly important, particularly for species that are commercially exploited and where management may need adjustment when factoring in global change effects (Denman *et al.*, 2011; Kjesbu *et al.*, 2014; Lam *et al.*, 2014). Studies on the effects of ocean acidification on larval fish growth have shown pronounced interspecies variability. For example, no effect on growth rate was observed for walleye pollack (*Theragra chalcogramma*) (Hurst *et al.*, 2013), whereas a decrease in growth rate under high pCO_2 was observed in Atlantic silverside (*Menidia beryllina*) (Baumann *et al.*, 2012) and summer flounder (*Paralichthys dentatus*) (Chambers *et al.*, 2013) compared to an increase in growth rate under high pCO_2 levels in cod (Frommel *et al.*, 2012) and sand smelt larvae (*Atherina presbyter)* (Silva *et al.*, 2016).

Currently, no generalizations with regard to ocean acidification effects on growth are possible among species. However, it is believed that enhanced growth results in higher survival during early-life-stages, due to less predation, since larvae outgrow the predator field faster (Bailey & Houde, 1989). This results in an evolutionary pressure for fast growth (Houde, 1997), which has been shown for certain Atlantic cod stocks (Meekan & Fortier, 1996). It is therefore increasingly important to understand the underlying mechanisms of ocean acidification leading to the effect on size and how trade-offs, made by the larvae, may manifest itself in changed growth patterns.

Although pollutants, low oxygen levels and inappropriate nutrition and temperature can affect organ and skeletal development with long-lasting effects on performance and fitness (Boglione *et al.*, 2013; Vanderplancke *et al.*, 2015) so far only limited information on ocean acidification effects on organ and skeletal development (Pimentel *et al.*, 2014a; Crespel *et al.*, 2017) and possible functional trade-offs are available.

Atlantic cod (*Gadus morhua*) is one of the most important commercial species and in particular the Arcto-Norwegian cod stock supports a large fishery. Their management is already affected by climate change (Kjesbu *et al.*, 2014). Using laboratory experiments we were able to show that larvae of the North-East Arctic cod stock show significantly increased mortality rates after hatching under end-of-the-century acidification levels with significant effects on population recruitment (Stiasny *et al.*, 2016; Königstein *et al.*, 2017). In the present study, we aim at further exploring whether the surviving larvae (35 to 36 dph) were affected by the acidification treatment. Therefore, we compared larvae fed with different prey densities to additionally analyse the effect of energy limitation from ambient (503 ppm)

and elevated CO_2 (1179 ppm) treatments and measured changes in size, dry weight, skeletal and organ development (liver, eyes, pancreas, kidney, gills). We discuss the potential trade-offs in growth and development in response to the different acidification treatments from two different feeding regimes.

Material 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 were 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. These 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). 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* and *Brachionus* until day 24, after which feeding was gradually switched to *Artemia* nauplii. Prey concentrations fed at each feeding were the same for both treatments, but number of feedings and therefore total amount fed differed (Table 1).

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 high 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.

Ambient (pCO_2: 503 ± 89 µatm; pH 7.9 ± 0.15) and increased CO_2 (pCO_2: 1179 ± 87 µatm; pH 7.61 ± 0.03) levels in the rearing tanks were achieved by controlling the pH values in a header tank with pH probes connected to an IKS computer system. If the values deviated from the set target pH, a magnetic valve was opened, which allowed a pulse of CO_2 from a storage bottle to go into the inflow of the header tank. 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 was furthermore manually checked every day in the rearing tanks with a separate pH/temperature probe (WTW pH/Cond 340i/3320). Water chemistry, including DIC and alkalinity, was tested weekly based on the Best Practices Guide (Riebesell *et al.*, 2010). For further details please consult the Supplementary Information of Stiasny *et al.* (2016).

For the growth measurements larvae were sampled alive, anaesthetized with MS222 (Ethyl 3-aminobenzoate methanesulfonate) and frozen at -20°C. 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).

Table 1: Details & concentrations of feeding schedule of both food treatments.

Low Food						High Food				
DPH	Greenwater/ *Nannochloropsis*	Rotatoria per tank per day (mill.)	Number of daily feedings of rotatoria	*Artemia* per tank per day (mill.)	*Artemia*: number of daily feedings	Greenwater/ *Nannochloropsis*	Rotatoria per tank per day (mill.)	Number of daily feedings of rotatoria	*Artemia* per tank per day (mill.)	*Artemia*: number of daily feedings
1	yes	4.27	7			yes	4.27	7		
2	yes	4.27	7			yes	4.27	7		
3	yes	4.27	7			yes	4.27	7		
4	yes	4.27	7			yes	4.27	7		
5	yes	7.35	7			yes	7.35	7		
6	yes	7.35	7			yes	7.35	7		
7	yes	7.35	7			yes	7.35	7		
8	yes	7.35	7			yes	7.35	7		
9	yes	7.35	7			yes	7.35	7		
10	yes	7.35	7			yes	7.35	7		
11	yes	7.35	7			yes	7.35	7		
12	yes	7.35	7			yes	7.35	7		
13		7.35	7				7.35	7		
14		3.15	3				7.35	7		

15		3.15	3				7.35	7		
16		3.15	3				7.35	7		
17		3.15	3				7.35	7		
18		3.15	3				7.35	7		
19		3.15	3				7.35	7		
20		3.15	3				7.35	7		
21		3.15	3				7.35	7		
22		3.15	3				7.35	7		
23		3.15	3				7.35	7		
24		3.15	3				7.35	7		
25		2.28	3	0.51	3		5.35	7	1.19	7
26		2.28	3	0.51	3		5.35	7	1.19	7
27		2.28	3	0.51	3		5.35	7	1.19	7
28		2.28	3	0.51	3		5.35	7	1.19	7
29		2.28	3	0.51	3		5.35	7	1.19	7
30				0.53	1				0.80	3
31				0.53	1				0.80	3
32				0.53	1				0.80	3
33				0.53	1				0.80	3
34				0.53	1				0.80	3
35				0.53	1				0.80	3

For the ossification analysis, 36 days old larvae were fixed in 70% ethanol (Schnell et al., 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 hours, the specimens were washed in a 70% ethanol solution to

remove excessive alcian blue and transferred for another 24 hours into 6 ml of a 0.5% KOH solution containing four droplets of a 3% H2O2 solution and alizarin red powder. The solution should have a dark purple coloration in order to obtain good staining results. During these 24 hours the tissues of the larvae were cleared by KOH, bleached by H2O2 and stained for calcified structures by alizarin red. Finally the specimens were transferred to a 70% glycerin solution for dissection and digital documentation. All specimens 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 was counted on these photographs. 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).

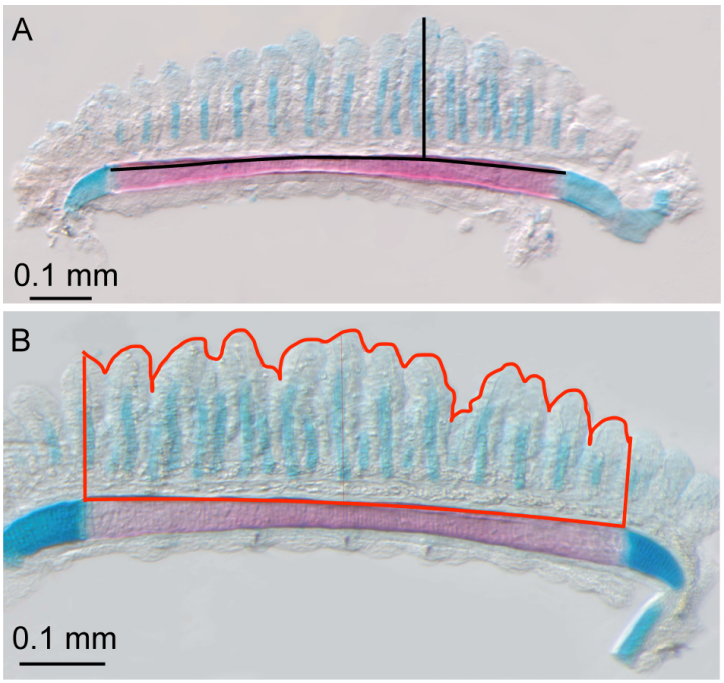

Figure 1: Third ceratobranchial with gill filaments of 36 days old cod larvae showing (a) the measured lengths of the ossified gill arch (horizontal line), which is stained in pink, and the longest gill filament (vertical line) and (b) the measured gill filament area (shown encircled in red).

Larvae for the histological analysis 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 3-category, qualitative score (some +/several ++/many +++). A similar score was used for registration of lipid vacuoles in the cod larvae livers.

RNA/DNA ratios reflect the relative condition and growth potential of fish larvae, due to the fact that while the DNA content is stable in the cell, the RNA content, representing the protein biosynthesis machinery of the cell, varies with the nutritional state of the larvae (Clemmesen, 1994). Prior to RNA/DNA analyses and lipid determination all larvae previously stored at -80°C were freeze dried for 16 hours (Christ Alpha 1-4 freeze dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany) and weighed (Sartorius SC2 microbalance, Sartorius AG, Göttingen, Germany; precision 0.1 µg). Measurements of RNA and DNA were made using the fluorometric 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 *et al.*, 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 hours. The defatted carcasses were transferred into Eppendorf vials and placed with open lids in a desiccator for 48 hours 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.

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.

For the analyses, two-way ANOVAs were used to test for interactions between the CO_2 and the food treatment. Transformations were used to achieve normality of the residuals whenever necessary and appropriate. Since several larvae per tank were used for analyses, the tank effect was assessed for every variable prior to analysis. However, the tank never had a significant effect on any of the parameters.

Results

Averaged over acidification treatments, larvae in the high food treatment were significantly longer (df=1, F=156.3, p<0.001) and heavier (df=1, F=277.6, p<0.001) than those in the low food treatment. Additionally, there was a significant interaction with the CO_2 treatment (SL: df=1, F=27.4, p<0.001; DW: df=1, F=16.99, p<0.001). While larval size at the high food regime was not affected by the acidification, larvae at the low food regime were significantly larger under high CO_2 compared to control CO_2 levels (Figure 2).

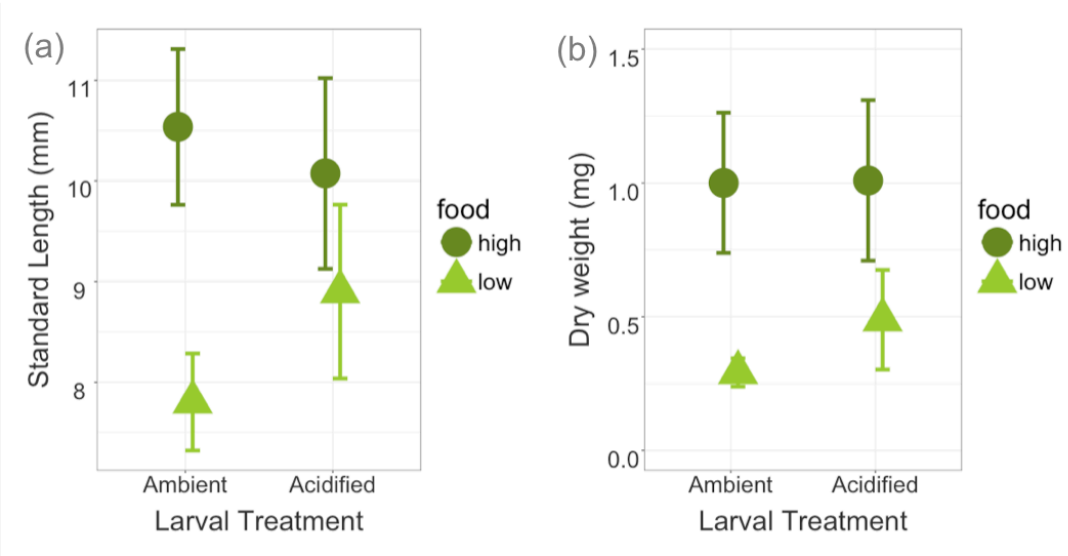

Figure 2: (a) Standard length of 36 days old cod larvae in mm and (b) Dry weight in mg. Shown are mean values +/- standard deviation. The darker circles show the high food treatment and lighter triangles the low food treatment. (N=11-26 per treatment)

Under ambient CO_2 larvae in the high food treatment had nearly 4-times more fully ossified vertebrae than those in the low food treatment. However, larvae in the low food, high CO_2 treatment had significantly more fully ossified vertebrae than those in the low food, ambient CO_2 treatment. In the high CO_2 treatment, the number of fully ossified vertebrae was similar between high and low food treatment larvae. (CO_2: df=1, F=13.1, p=0.001; food: df=1, F=21.2, p<0.0001; CO_2*food: df=1, F=26.3, p<0.0001) (Figure 3)

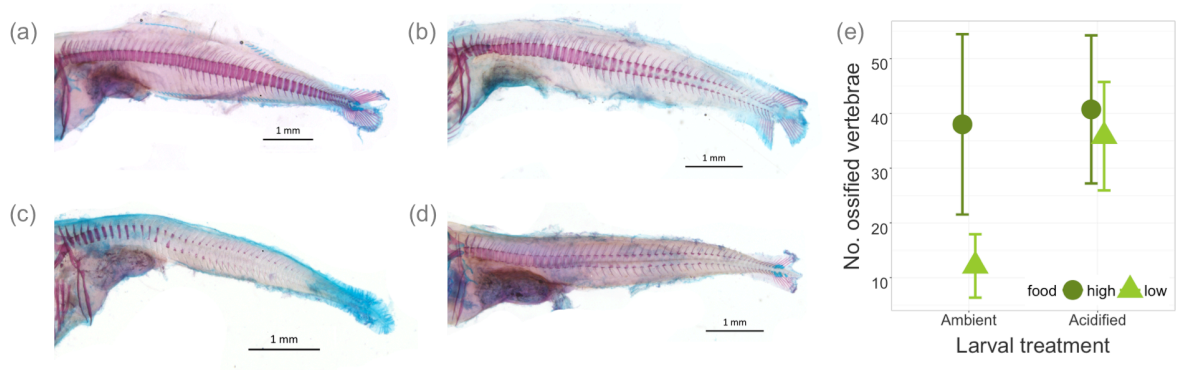

Figure 3: Image of cleared and double stained specimens showing number of ossified vertebrae (coloured in red) in 36 days old cod larvae in (a) ambient CO_2, high food (b) acidification, high food (c) ambient CO_2, low food (d) acidification, low food. Mean number (+/- standard deviation) of fully ossified vertebrae per treatment shown in (e). The darker circles show the high food treatment and lighter triangles the low food treatment. (N=7-12 per treatment)

The RNA/DNA ratios were not significantly different between the CO_2 treatments under high food regime, but did differ between food regimes (df=1, F=244.4, p<0.0001) with overall lower RNA/DNA ratios in the low food treatment. The interaction between treatments was also significant (df=1, F=5.4, p=0.02). Larvae in the acidified treatment showed higher RNA/DNA ratios than those in the ambient CO_2 treatment under low food conditions (Figure 4a). The lipid content (Figure 4b) was on average between 18 and 19% in all larvae from the high 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 (interaction CO_2*food: df=1, F=13.6, p<0.001).

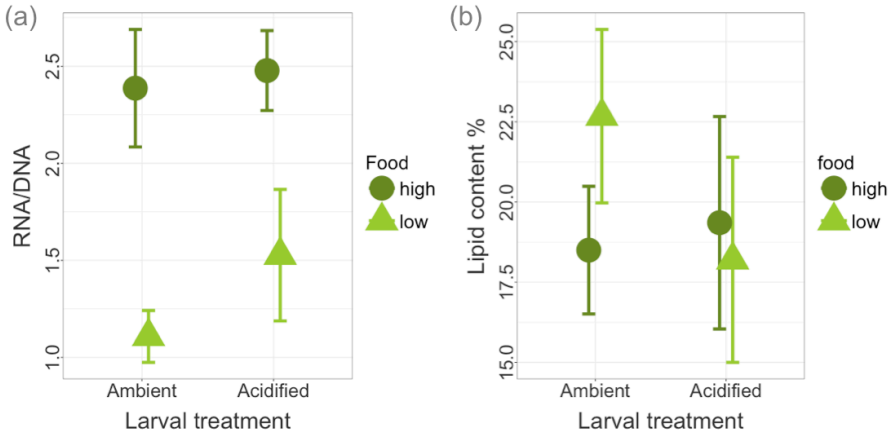

Figure 4: (a) RNA/DNA content of the 36 days old cod larvae (N=10-18 per treatment) and (b) Lipid content of the larvae (N=11-19 per treatment; mean values +/- standard deviation). The darker circles show the high food treatment and lighter triangles the low food regime.

The length of the ossified gill arches was significantly affected by the food regime (df=1, F=21.9, p<0.001) with longer ossified arches in the high food regime (Figure 5a). The CO_2 treatment showed a significant interaction (df=1, F=8.1, p<0.01) with food regime. Larvae at low food had longer ossified arches in the high CO_2 treatment than those in the ambient treatment. The length of the longest gill filament was significantly affected by both treatments (CO_2: df=1, F=22.1, p<0.001; food: df=1, F=5.9, p=0.02), with no significant interaction between the two factors. The gill filament was always longer in the ambient CO_2 treatment and in the high food regime. (Figure 5b) The gill area was significantly affected by both treatments and their interaction (CO_2: df=1, F=11.3, p<0.01; food: df=1, F=14.9, p<0.001; interaction CO_2*food: df=1, F=8.8, p<0.01) with larger gill areas shown by the larvae in the high food compared to the low food regime in the larvae in the ambient CO_2 treatment. Larvae in the high CO_2 treatment were always similar to those at ambient CO_2, low food treatment independent of their own food treatment. (Figure 5c)

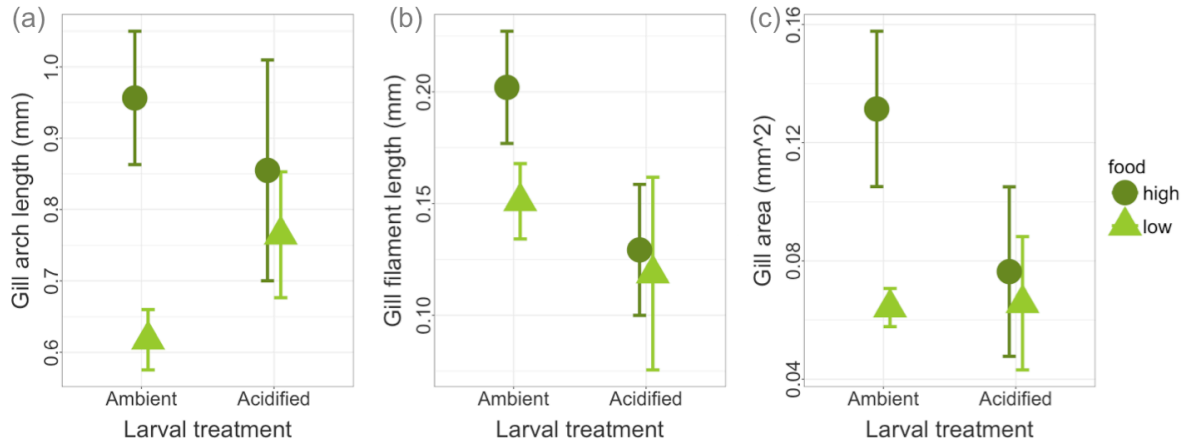

Figure 5: (a) Length of the ossified gill arch (mm) of 36 days old cod larvae (b) length of the longest gill filament (mm) (c) gill area (mm^2), mean values +/- standard deviation. The darker circles show the high food treatment and lighter triangles the low food treatment. (N=5-8 per treatment)

The histological samples investigated showed different severities of impairments in the organs. While vacuoles in the eyes were found across all treatments, vacuoles in the pancreas, the kidneys and particularly in the liver were found most commonly in larvae in the acidified, low food regime. Impairments in the pancreas and the kidneys were mild, but larvae from the acidified, low food treatment showed partly severe vacuolization. (Figure 6)

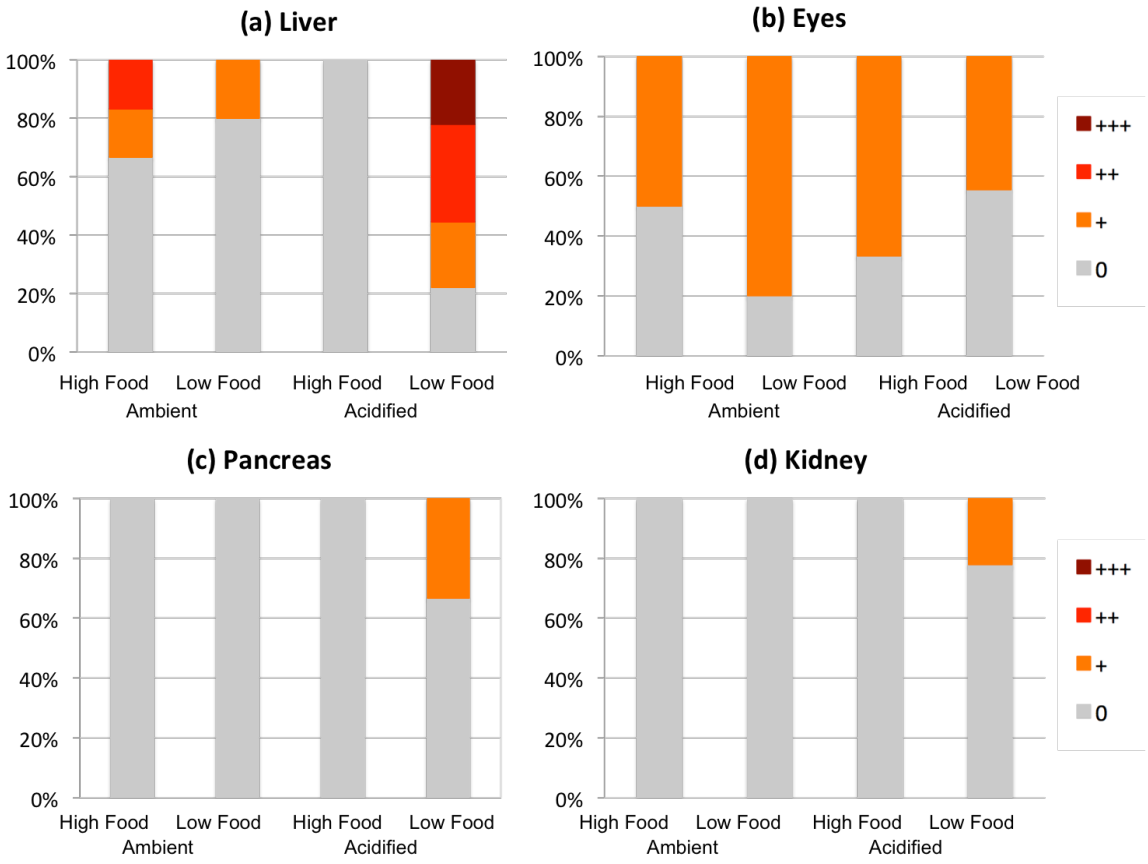

Figure 6: Frequency of organ impairments in (a) liver, (b) eyes, (c) pancreas, and (d) kidney of the 35 days old cod larvae in a subjective scale from 0 (no impairments) to +++ (very severe/many impairments). (N=3-9 per treatment)

Discussion

Under realistic end-of-the-century CO_2 concentrations (Denman *et al.*, 2011; AMAP, 2013) a significant interaction between prey availability and acidification on the growth and development of the Arcto-Norwegian cod larvae became apparent. The high food regime provided prey organisms *ad libitum*, which resulted in larger larvae in better nutritional condition, as evident by the higher RNA/DNA ratios. Nearly all vertebrae were ossified at 36 days post-hatching in the high food treatment and increased CO_2 concentration did not result in significant ossification differences (e.g. length of the ossified gill arches). The lipid content in the larvae did not differ either.

However, larvae in the low food treatment showed significant differences according to the CO_2 treatment. Larvae from the acidified treatment 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, under low food availability. This result is unexpected since these larvae are energetically more limited. In fact, larvae in the acidified treatment showed less difference between food regimes compared to the ambient

CO_2 treatment. Larvae from the low food treatment exhibited similar growth and skeletal development as those raised under high food when experiencing high CO_2 concentrations. A noticeable exception is found in organ development. Particularly the liver is heavily impaired in the low food, acidified treatment, but not in the high food, acidified treatment. One possible explanation is the trade-off that the larvae in the low food treatment ultimately have to make since they are energy limited. Apparently, they spend more energy on growth and ossification of skeletal elements in the acidified treatment compared to larvae in the ambient treatment. The lower lipid content might be associated with ossification, since dietary lipids are important energy stores for skeletal development in marine fish (Lall & Lewis-McCrea, 2007; Kjørsvik *et al.*, 2009). Additionally, these lipid levels might indicate that larvae at low food, ambient CO_2 treatment are still in an earlier developmental stage compared to larvae from the other treatments. This is also supported by the smaller larval size and fewer ossified vertebrae in this treatment. Similar organ impairments of liver and gills have been already documented as a downside of the trade-off in cod, herring, tuna and summer flounders (Frommel *et al.*, 2012, 2014, 2016; Chambers *et al.*, 2013). The gill size was always smaller in the acidified treatment, independent of the food regime, even though larval size was increased in the low food treatment. 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 *et al.*, 2017), these differences likely result in a very significant difference in functionality. The apparent decoupling of larval size and gill size may have a huge effect on the fitness of the larvae in the following weeks to months.

Studies on the effects of ocean acidification on marine fishes have steadily increased, however the available data on growth and development in marine temperate fish species do not allow for a general conclusion. This is due to different experimental setups, different analyzed life stages (often limited to very early life stages, like embryos, non-feeding larvae), and short-term versus long-term effects. Several studies have found negative effects of ocean acidification on the size of larval fishes. Only few studies have looked at the effect of ocean acidification on the whole body and organ level in relation to prey density. Baumann *et al.* (2012) found reduced standard lengths in the Atlantic silverside larvae (*Menidia beryllina*) with increasing acidification levels. A similar negative correlation between CO_2 concentration and larval size was found for the yellowfin tuna (*Thunnus albacares*) (Bromhead *et al.*, 2015) and Atlantic herring (*Clupea harengus*) (Frommel *et al.*, 2014). Other species, like juvenile scup (*Stenotomus chrysops*) (Perry *et al.*, 2015) or walleye pollock larvae (*Theragra chalcogramma*) (Hurst *et al.*, 2013) showed little to no effect of CO_2 on growth. Frommel *et al.* (2012) observed increased larval growth in Atlantic cod during some period of development, though at much higher CO_2 concentrations of above 4000 µatm. The orange clownfish *Amphiprion percula* similarly showed increased larval growth until settlement under increased CO_2 concentrations, although this effect was not observed consistently in larvae from different parents (Munday *et al.*, 2009). Crespel et al. 2017 demonstrated a limited influence of end-of-century CO_2 levels on growth and survival in sea bass larvae, but also showed faster skeletal development under acidified conditions.

When interpreting the results on growth and development, it is important to keep in mind that survival in these larvae was found to be significantly lower under the elevated CO_2 treatment, irrespective of food levels (Stiasny et al. 2016). It is plausible that an increased mortality under elevated CO_2 levels led

to a selection of specific phenotypic traits, such as for higher growth. It remains hypothetical though whether selection would be stronger under limited prey availability, which is suggested by significant differences in larval length between CO_2 treatments at low food but not at high food levels. It was shown before that unfavourable prey availability could lead to a selection for phenotypes with higher growth (Meekan & Fortier, 1996; Folkvord *et al.*, 1997) (Meekan et al. 1996, Folkvord et al. 1997). Unfortunately, this question cannot be answered with the data originating from this study, but is definitely an important issue to consider for future experiments.

Increased calcification of the otoliths due to acidification has been shown for some species (Hurst *et al.*, 2012; Bignami *et al.*, 2013; Maneja *et al.*, 2013; Pimentel *et al.*, 2014b; 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 made of calcium phosphate, not calcium carbonate like the otoliths. The exact interaction remains unknown, but 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). Whereas Pimentel *et al.* (2014) found skeletogenesis to be defected in the larvae of *Solea senegalensis*. In contrast to the cod larvae in this study, a large proportion of the larvae of *S. senegalensis* showed severe deformities under increased CO_2 concentrations at a similar level.

While our study confirms increased larval size under ocean acidification - but only in an energy limited situation - it is premature to associate this finding with increased fitness of larvae. To the contrary, the full picture of developmental processes is far more complicated, and larger larvae may develop more subtle bone and organ damage. Increased larval size may result in developmental patterns that can possibly decrease fitness of the larvae through decreased functionality of the organs or even impairments. So far it remains unclear which factors drive such hypothesized trade-offs and causes the larvae to invest so heavily into growth.

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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

The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.C.

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CHAPTER 2

TRANSCRIPTOME PROFILING REVEALS EXPOSURE TO PREDICTED END-OF-CENTURY OCEAN ACIDIFICATION IS A STEALTH STRESSOR FOR ATLANTIC COD LARVAE

Transcriptome profiling reveals exposure to predicted end-of-century ocean acidification is a stealth stressor for Atlantic cod larvae

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Abstract

Ocean acidification (OA), caused by the uptake of increasing atmospheric CO_2 concentration by the oceans, is impacting many fish species, but little is known about the underlying molecular responses of observed physiological impacts. In this study, we characterized the whole transcriptome of larval stages of Atlantic cod (*Gadus morhua*) exposed to simulated OA at levels (1100 µatm CO_2 (global end-of-century prediction IPCC RCP 8.5) vs. ambient 500 µatm CO_2) known to induce tissue damage and elevated mortality. Intriguingly, only few genes were differentially expressed in 6 and 13 days-post-hatch larvae (3 and 16 genes, respectively) at a time point when the maximal mortality in the treatment group occurred. At 36 dph, 1413 genes were differentially expressed, most likely caused by developmental asynchrony between the treatment groups, with OA treated individuals displaying faster growth. A target gene analysis revealed only few genes of the cellular stress response to be differentially expressed.

In summary, our results show no clear divergence in gene expression patterns in early larvae exposed to increased pCO_2 levels, which are in strong contrast to the observed influence on fitness related measurements. We thus suggest that predicted ocean acidification levels constitute a “stealth stress” for early Atlantic cod larvae.

Introduction

Global change, caused by diverse anthropogenic activities, is the defining characteristic of the Anthropocene [1] and has already started to affect marine ecosystems (reviewed in [2]). One of the major threats of climate change (as part of global change) is ocean acidification (OA). It is caused by the uptake of rising atmospheric CO_2 concentrations by ocean waters, which is driven by fossil fuel burning and altered land use, lowering the ocean pH as well as the carbonate saturation state [3]. These changes have been shown to impact marine ecosystems [3–5], and particularly calcifying species including foundation species such as corals [6]. For marine fish species, adults are less susceptible to increased pCO_2 compared to early life stages [7–9]. Due to their high capability of acid-base regulation they can tolerate pCO_2 levels far exceeding [10], what is projected for the near future [11]. During early life stages exposure to increased pCO_2 has been shown to induce severe effects such as decreased hatching success [9], increased larval mortality [8], changes in growth and developmental patterns [7,12,13], decreased oxygen consumption rate [14] and impaired sensory abilities and behaviour [15,16]. Further, changed otolith and bone development [13,17,18] as well as tissue and developmental damages [13,19,20] have been observed.

The regulatory mechanisms and capabilities of adult fish to achieve and sustain acid-base balance have been identified [21–23], but very little is known about acid-base regulation in early life stages. Gill epithelia, the most important tissue for acid-base regulation in adult fish [21], are only developing in larvae, with a complete absence at hatch in cod larvae (for cod see [24]). During early development, all proton excretion occurs via chloride cells (Na^+/K^+ ATPase-rich ionocytes) located in the skin [25] and the primordial gill cavity [9]. The detrimental effects of OA on larvae are most likely due to the organisms' limited ability to regulate their acid-base balance or the cost of increased regulation. The increased cost of acid-base regulation in an acidified ocean could have potential impacts on fitness [13,23,26].

With the increasing availability of next generation sequencing techniques, global transcriptome profiling in experimental settings with non-model organisms has become possible. This allows to connect environment and genotype and thus, elucidate the molecular basis for observed phenotypes [27] . There are, so far, few attempts on untangling the full transcriptomic response to ocean acidification stress in fish. While most studies are based on candidate gene approaches concentrating on acid-base regulation [28,29], more recent studies have, however, employed whole transcriptome sequencing methods (for a review see [30]) and identified differentially expressed (DE) genes. In these studies the focus was mainly on the cellular stress response [31] and neurological signalling in adult fish [32]. Underlying transcriptomic mechanisms of above mentioned effects during early development remain unclear, and thus, even fewer studies have focused on the gene expression changes to ocean acidification in eggs and larvae [12].

Ocean acidification, as well as changes in temperature and hypoxia can impact the function of many cellular processes and cause damages to cellular macromolecules such as DNA and proteins. In order to avert damage, cells can react to harmful changes in their cellular environment by means of the cellular stress response (CSR); defined by Kültz [33].The CSR is manifested as the minimal stress proteome, consisting of circa 300 proteins with a core number of highly conserved genes throughout all organisms [34]. Proteins of the CSR are involved in, but not limited to, functions in molecular chaperoning, DNA repair, protein folding, redox regulation and energy metabolism. Generally, cells initiate a CSR in

response to damaged macromolecules by increasing transcription of the genes encoding for the stress proteome. This leads to increased concentration of CSR proteins while their activity is further controlled by post translational modifications [34]. Increased pCO_2 levels are known to induce differential expression of genes of the CSR in tissues of adult fish [31] but again very little is known about the CSR in larval fish under the stress that is imposed by increased pCO_2.

This study employed global mRNA sequencing (RNA-Seq) to assess the transcriptomic response of Atlantic cod (*Gadus morhua*) larvae, a key species of ecological and economic importance in the North Atlantic, to global mean ocean acidification levels predicted for end-of-century, according to RCP 8.5 [11] compared to present day levels. However, regionally these levels will most likely to be reached earlier [35]. In the experimental set-up, Atlantic cod embryos and subsequent larvae were exposed to either ambient or end-of-century pCO_2 concentrations (see [8] for more detailed information). In these studies, predicted ocean acidification was shown to induce dramatic consequences for fitness related measurements, i.e. a doubling in the daily mortality rate [8] and other severe phenotypic differences like changed ossification speed in vertebrae, gill development and histological tissue changes such as liver vacuolization [13]. For the transcriptome analysis, special emphasis was placed on gene families connected to the CSR to address the question if Atlantic cod during early developmental stages show signs of cellular stress in response to ocean acidification.

Results

Larval dry weight

The early two larval stages, 5 (Figure 1a) and 15 days-post-hatch (dph) (Figure 1b), showed no differences in dry weight between ambient and high pCO_2 treatment (DF=1, F=6.00, p=0.08 and DF=1, F=2.06, p= 0.25, respectively) (full results SI Table 1). A significant difference in dry weight was observed at 36 dph (Figure 1c), where larvae from the high pCO_2 treatment were significantly heavier than larvae from ambient pCO_2 levels (presented in [13]).

Whole transcriptome sequencing

Illumina sequencing generated a total of $3.4 * 10^9$ raw reads (sample average $35.5 * 10^7$ read-pairs 151 bp in length) with a range of $3.07 - 6.61 * 10^7$ reads. After adaptor trimming and discarding unpaired reads, an average of $3.52 * 10^7$ paired reads per biological replicate remained (Table 1). On average, 69.2 % of reads per sample could be uniquely assigned to the transcriptome generated from the annotated genome.

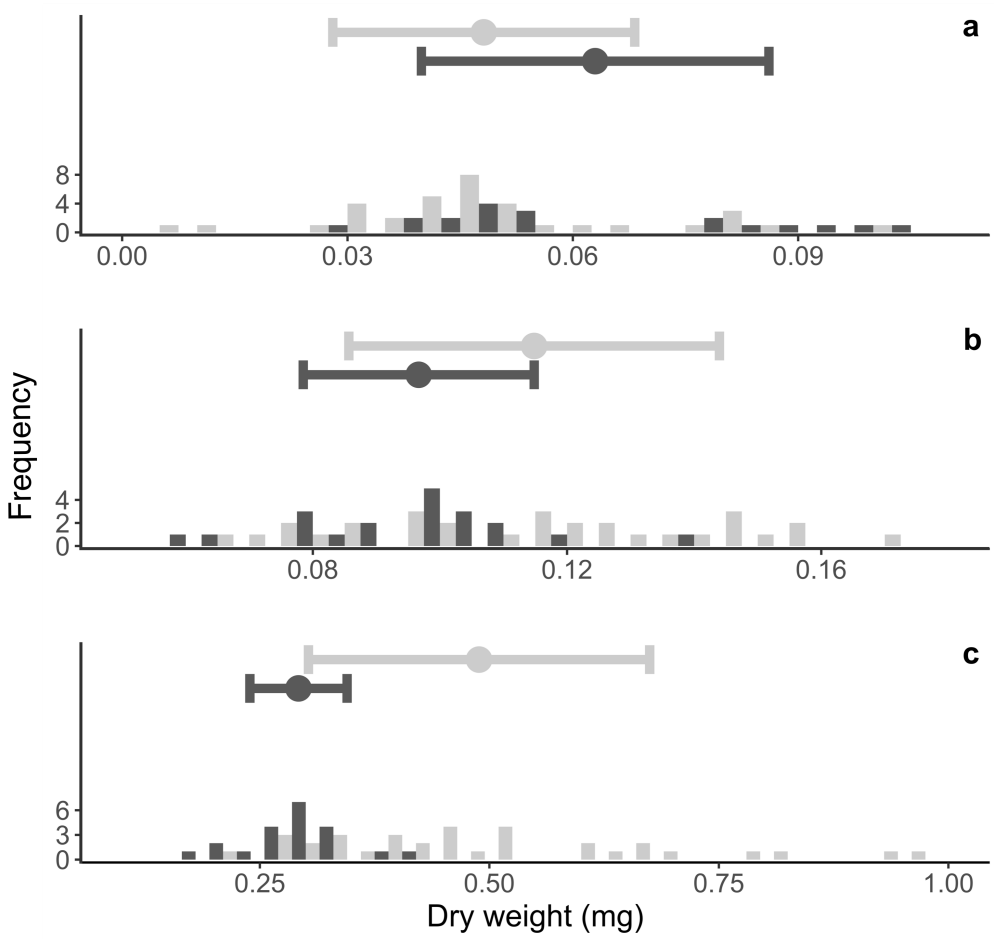

Figure 1: Means and histograms of larval dry weight in mg at a) 5, b) 15 and c) 36 dph (modified from Stiasny et al., submitted). Grey boxes correspond to larvae from the high pCO_2 (~1179 µatm) treatment, black boxes correspond to larvae from the ambient (~503 µatm) treatment. The centre represents the mean, the whiskers standard deviation (SD). Please note the difference in the frequency and dry weight scales.

Table 1: Summary of samples, treatments, sequencing and assignment results. # presents number, SD is standard deviation. Read values refer to single reads, raw reads are total number of reads per sampling category, and filtered reads are average per sample.

			RAW READS DE-MULTIPLEXED			FILTERED READS USED IN DEANALYSIS		ASSIGNED UNIQUELY TO THE REF. TRANSCRIPTOME	
Treatment	Age (dph)	# Samples	Sum	Mean #	SD	Mean #	SD	Mean %	SD
Ambient pCO_2	6	8	$5.1*10^8$	$6.4*10^7$	$1.8*10^5$	$6.4*10^7$	$1.8*10^5$	70.58	1.00
High pCO_2	6	8	$5.3*10^8$	$6.6*10^7$	$2.8*10^5$	$6.5*10^7$	$2.8*10^5$	70.52	0.96
Ambient pCO_2	13	8	$6.2*10^8$	$7.8*10^7$	$24.3*10^5$	$7.7*10^7$	$24.2*10^5$	71.11	0.75
High pCO_2	13	8	$5.2*10^8$	$6.5*10^7$	$4.8*10^5$	$6.4*10^7$	$4.7*10^5$	71.29	1.35
Ambient pCO_2	36	8	$6.9*10^8$	$8.6*10^7$	$28.5*10^5$	$8.4*10^7$	$28.2*10^5$	61.23	2.88
High pCO_2	36	8	$5.4*10^8$	$6.7*10^7$	$2.8*10^5$	$6.7*10^7$	$2.7*10^5$	70.51	2.92
Total		48	$34.1*10^8$	$7.1*10^7$		$7.0*10^7$		69.20	

Larvae sampled at 6 dph revealed only three DE genes (p_{adj} <0.05, FDR=BH) (SI Table 2). All three genes were downregulated in the high pCO_2 treatment compared to the ambient treatment. A similar result was found for the 13 dph larvae, where a total of 16 genes was differentially expressed but unlike the youngest larvae, 7 genes were upregulated under high pCO_2 (SI Table 3). This low transcriptomic response at the first two early larval stages stood in strong contrast to the response manifested in the expression patterns of the oldest group as visualized in the fold change/mean counts plots (MAplots) (Figure 2). In 36 dph larvae, 1413 genes were differentially expressed, 19 of which were up- or down-regulated by a factor of 2 or more (SI Table 4).

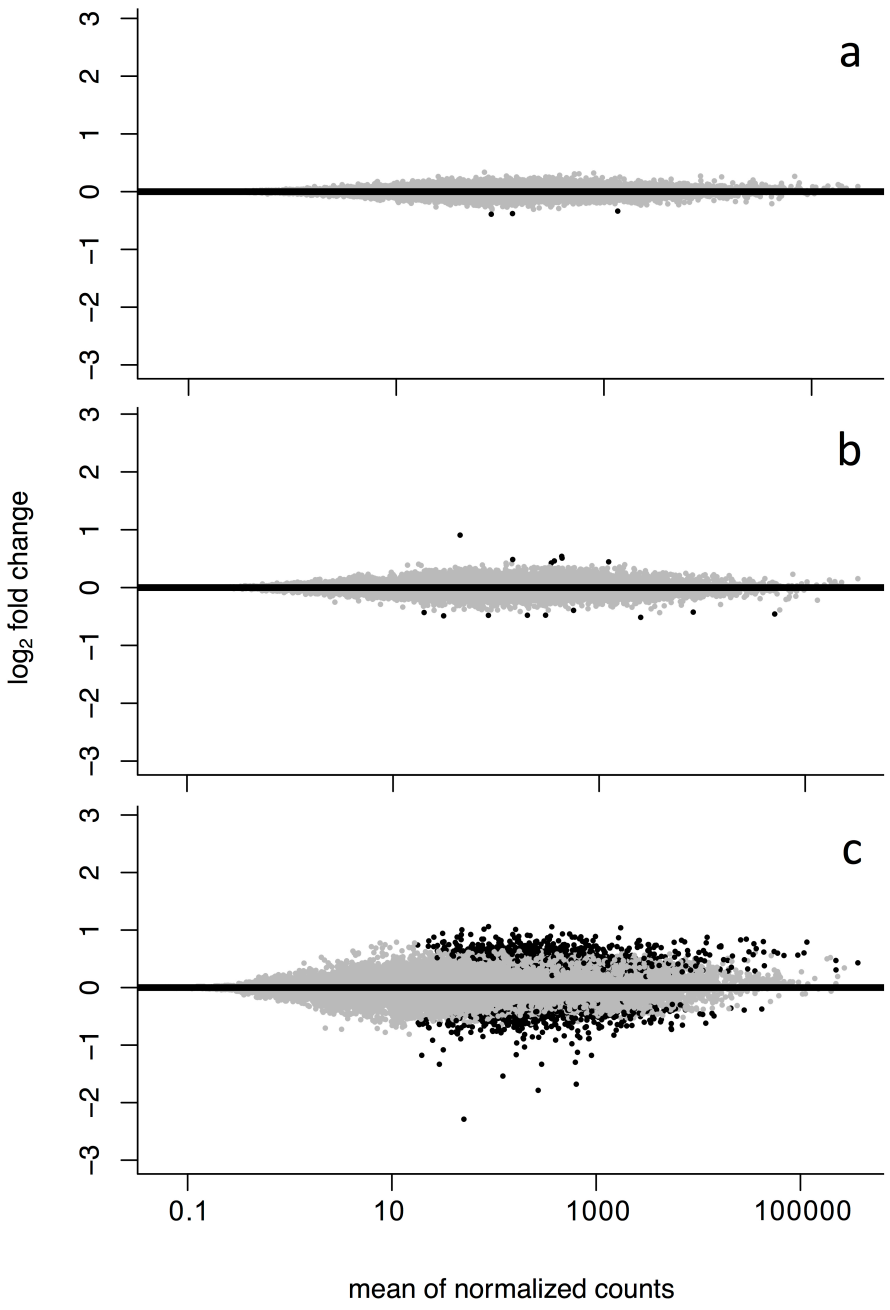

Figure 2: MA plots of log2 fold change against mean of normalized counts for a) 6 dph b) 13 dph and c) 36 dph larvae. Black dots represent genes significantly differential expressed (padj<0.05, false discovery rate correction (FDR)=Benjamini-Hochberg(BH)) between ambient (~503 µatm) and high (~1179 µatm) pCO_2 treatment.

This pattern between the treatments at different ages was visualized in a principal component plot (Figure 3, PC1 = 50 %, PC2 = 7.2 %). All three age groups were well separated by the 1st and 2nd component of the PCA representing differences in gene expression patterns between age groups (ANOSIM 999 permutations, R = 0.799, p = 0.001). For young larvae (6 and 13 dph), replicates did not

segregate according to pCO_2 treatment, indicating only small differences in transcriptomic response to ocean acidification. 36 dph replicates exhibited a large spread along both components and were at least partially clustered by treatment reflecting the large number of DE genes. Further, none of our candidate genes, both in the CSR or acid-base regulation were identified in the 25 main contributing genes to loadings in the first and second principle component (Table 2a and 2b).

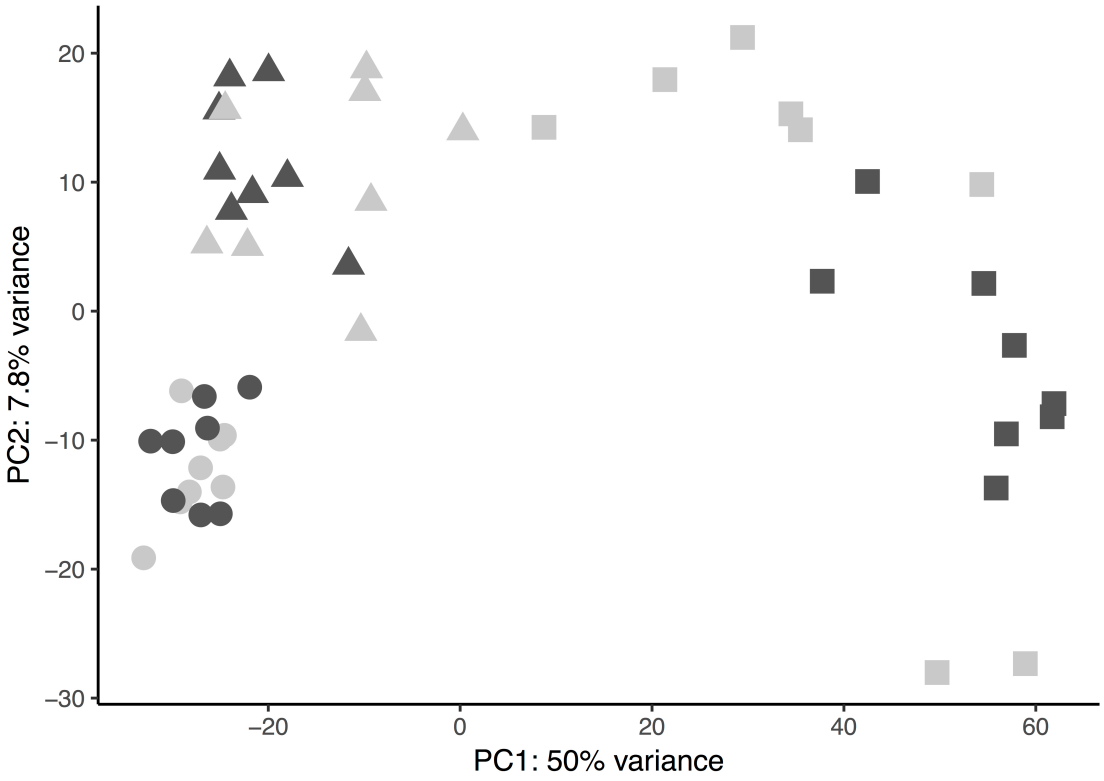

Figure 3: Principle component analysis of total gene expression profile, visualized for first and second component. Shapes represent larval age groups, circle 6 dph, triangle 13 dph and, squares 36 dph, while colours correspond to larval treatment, black ambient (~503 µatm) and grey high (~1179 µatm) pCO_2

A gene ontology enrichment analysis, performed on the 1413 differentially expressed genes in 36 dph cod larvae yielded two significantly enriched GO terms ($p_{adj.}$<0.05, FDR=BH): extracellular matrix (GO:0031012) and mitotic chromosome condensation (GO:0007076). GO terms related to the CSR such as “response to stress” GO:0006950 and its children terms including “cellular response to stress” GO:0033554 were not enriched. Further, no GO terms related to acid-base regulation, for example “proton transport” GO: 0015992 or “bicarbonate transmembrane transporter activity” GO:0015106, were significantly enriched.

None of the genes belonging to the cellular stress response (CSR) ((34), table 1)were differentially expressed at 6 or 13 dph. Also, none of the pre-defined candidate genes encoding important enzyme components of acid base regulation, for example Na^+/H^+ exchanger, HCO_3^- transporters and carbonic anhydrase among others [22,28] were differentially expressed at these ages. None of the differentially expressed genes could be associated to cell or protein damage.

In 36 dph cod larvae, on the other hand, a number of genes belonging to 10 of 45 CSR gene families, as well as additional heat shock proteins and genes related to acid-base regulation were differentially expressed, but at low fold changes. Heat shock protein 70 kDa (HSP70) was present in several variants in the reference transcriptome, one of which was upregulated by a fold change of 1.68 (l2FC = 0.75, p<0.05). In addition, Serpin, also known as heat shock protein 47 kDa (HSP47), was moderately upregulated in the high pCO_2 treatment (l2FC = 0.72, p<0.01). Additionally, a number of DNA J homologs (Dnajc9 and Dnajb6-b) were differentially expressed (l2FC = 0.53 and 0.61, p<0.05 and p<0.05, respectively). Upregulated genes related to the CSR further include: one of two homologs of Seratin proteases HTRA3 and HTRA1A (l2FC = 0.63, p<0.05 and l2FC=0.49, p<0.01 respectively), Long-chain-fatty-acid CoA ligase 6 ACSL6 (l2FC = 0.5, p<0.05), Beta-enolase ENO3 (l2FC = 0.51, p<0.05), Propyl endopeptidase Prep (l2FC = 0.34, p<0.05), MutS/MSH protein homolog 4 MSH4 (l2FC = 0.38, p<0.05), Aldo-keto reductase AKR1B10 (l2FC = 0.23, p<0.05), Aldehyde dehydrogenase ALDH1A3 (l2FC = 0.39, p<0.05), Nucleoside diphosphate kinase NME1-2 (l2FC = 0.60, p<0.01) and Glycerol-3-phosphate dehydrogenase gpd1 (l2FC = 0.85, p<0.01).

Of 45 gene families in the highly conserved CSR, five families or genes were not present in the available annotations or were not expressed in the samples, leading to a total of 185 annotations related to the CSR of which 14 were differentially expressed (p_{adj} (FDR=BH) ≤ 0.05). Genes coding for HSP60 chaperonin, Peroxiredoxin, Superoxide dismutase, Thioredoxin, RAD51 DNA repair proteins, Lon protease, Long-chain- fatty acid ABC transporters and others were expressed in both treatments with no significant differences.

Of 35 identified candidate genes and their variants directly involved in acid-base regulation, only one gene was differentially expressed. One of three annotations encoding carbonic anhydrase 4 (CA4) was downregulated in the high *p*CO2 treatment (l2FC=-0.54, p<0.01). Further, a number of ammonium transport rhesus (Rh) proteins, involved in CO_2 transport across membranes, were differentially expressed. One Ammonium transporter, namely Rh type C2 (rhgc2) was down regulated (l2FC=-0.37, p<0.05) while one of two annotations of Ammonium transporter Rh type B (rhbg) was up regulated (l2FC =-0.40, p<0.05) in the high pCO_2 treatment. Only one of the genes directly involved in acid-base

regulation, Pendrin (SLC26a4), was not identified in the reference genome. Of 41 annotations related to acid-base regulation only one (CA4) was differentially expressed between the treatments. Of the ammonium transport Rh proteins, two out of seven were expressed at significantly different levels. Further none of the candidate genes are present in 25 main contributing genes to the first (Table 2a) and second principle component (Table 2b) in figure 3.

Table 2a: Loadings of first principal component, 25 main contributing genes to first principle component in Figure 3

GENE	CONTRIBUTION TO PC 1 IN %
MYOSIN HEAVY CHAIN. FAST SKELETAL MUSCLE (*CYPRINUS CARPIO*)	0.388066021
KLHL38 KELCH-LIKE PROTEIN 38 (*DANIO RERIO*)	0.371193025
FBXO32 F-BOX ONLY PROTEIN 32 (*SUS SCROFA*)	0.302487707
MYOSIN HEAVY CHAIN. FAST SKELETAL MUSCLE (*CYPRINUS CARPIO*)	0.301755979
PROTEIN OF UNKNOWN FUNCTION	0.237395845
GAMMA-CRYSTALLIN M3 (*CYPRINUS CARPIO*)	0.223435332
PROTEIN OF UNKNOWN FUNCTION	0.218408605
LADDERLECTIN (*ONCORHYNCHUS MYKISS*)	0.217564849
FAM134B RETICULOPHAGY RECEPTOR FAM134B (*MUS MUSCULUS*)	0.198892372
PROTEIN OF UNKNOWN FUNCTION	0.182636968
PROTEIN OF UNKNOWN FUNCTION	0.181408566
MYOSIN HEAVY CHAIN. FAST SKELETAL MUSCLE (*CYPRINUS CARPIO*)	0.175043319
ARRDC2 ARRESTIN DOMAIN-CONTAINING PROTEIN 2 (*HOMO SAPIENS*)	0.170943549
IGFBP1 INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN 1 (*BOS TAURUS*)	0.170560436
VCAN VERSICAN CORE PROTEIN (*GALLUS GALLUS*)	0.168464054
EXOSC6 EXOSOME COMPLEX COMPONENT MTR3 (*DANIO RERIO*)	0.162277631
UCP2 MITOCHONDRIAL UNCOUPLING PROTEIN 2 (*MUS MUSCULUS*)	0.161029997
VISININ (*GALLUS GALLUS*)	0.160272963
BNIP3 BCL2/ADENOVIRUS E1B 19 KDA PROTEIN-INTERACTING PROTEIN 3 (*MUS MUSCULUS*)	0.159401846
VERRUCOTOXIN SUBUNIT BETA (*SYNANCEIA VERRUCOSA*)	0.154615965
C4 COMPLEMENT C4 (*RATTUS NORVEGICUS*)	0.15237641
HMGCS1 HYDROXYMETHYLGLUTARYL-COA SYNTHASE. CYTOPLASMIC (*GALLUS GALLUS*)	0.1502471
COL9A3 COLLAGEN ALPHA-3(IX) CHAIN (*GALLUS GALLUS*)	0.149620902
CYP51A1 LANOSTEROL 14-ALPHA DEMETHYLASE (*MACACA FASCICULARIS*)	0.145205011

Table 2b: Loadings of first principal component, 25 main contributing genes to second principle component in Figure 3

GENE	CONTRIBUTION TO PC 2 IN %
ES1 ES1 PROTEIN. MITOCHONDRIAL (*DANIO RERIO*)	0.631738076
MYOSIN HEAVY CHAIN. FAST SKELETAL MUSCLE (*CYPRINUS CARPIO*)	0.518263622
MRC1 MACROPHAGE MANNOSE RECEPTOR 1 *(HOMO SAPIENS*)	0.357842258
HCEA HIGH CHORIOLYTIC ENZYME 1 (*ORYZIAS LATIPES*)	0.315547116
PROTEIN OF UNKNOWN FUNCTION	0.292950430
IMPA1 INOSITOL MONOPHOSPHATASE 1 (*BOS TAURUS*)	0.284643925
C3 COMPLEMENT C3 (FRAGMENT) (*ONCORHYNCHUS MYKISS*)	0.282401366
COL10A1 COLLAGEN ALPHA-1(X) CHAIN (*GALLUS GALLUS*)	0.246564047
EXOSC6 EXOSOME COMPLEX COMPONENT MTR3 (*DANIO RERIO*)	0.228810163
PROTEIN OF UNKNOWN FUNCTION	0.201614736
PROTEIN OF UNKNOWN FUNCTION	0.186421801
PEG3 PATERNALLY-EXPRESSED GENE 3 PROTEIN (*BOS TAURUS*)	0.179113111
ELASTASE-1 (*SALMO SALAR*)	0.179070813
RPL31 60S RIBOSOMAL PROTEIN L31 (*PARALICHTHYS OLIVACEUS*)	0.177788248
ENTPD5 ECTONUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE 5 (*GALLUS GALLUS*)	0.177782763
COL1A2 COLLAGEN ALPHA-2(I) CHAIN (*RATTUS NORVEGICUS*)	0.176887258
VCAN VERSICAN CORE PROTEIN (*GALLUS GALLUS*)	0.174817539
CKMT1 CREATINE KINASE U-TYPE. MITOCHONDRIAL (*GALLUS GALLUS*)	0.173735764
LECT2 LEUKOCYTE CELL-DERIVED CHEMOTAXIN-2 (*HOMO SAPIENS*)	0.169742346
CELA3B CHYMOTRYPSIN-LIKE ELASTASE FAMILY MEMBER 3B (*MUS MUSCULUS*)	0.164459664
INTERMEDIATE FILAMENT PROTEIN ON3 (*CARASSIUS AURATUS*)	0.164073293
PROTEIN OF UNKNOWN FUNCTION	0.163405256
C3 COMPLEMENT C3 (FRAGMENT) (*ONCORHYNCHUS MYKISS*)	0.158777821
ITIH3 INTER-ALPHA-TRYPSIN INHIBITOR HEAVY CHAIN H3 (*MUS MUSCULUS*)	0.156855282

Discussion

In this study we uncovered no clear divergence in gene expression patterns in Atlantic cod larvae exposed to increased pCO_2 levels, which are in strong contrast to the observed influence on fitness related measurements, such as growth and survival [8,13].

Only very few genes were differentially expressed in young Atlantic cod larvae (6 and 13 dph) in response to simulated ocean acidification. This is all the more surprising as this was the phase when increased mortality occurred in response to increased pCO_2 [8]. However, older larvae (36 dph) showed a much larger number of genes differently expressed between ambient (~503 µatm) and high (~1179 µatm) pCO_2 levels. This difference could be attributed to the observed difference in growth [13], and thus the potential divergence in development, between larvae from the respective pCO_2 treatments. Furthermore, during the early larval stages no differential expression of candidate genes related to acid-base regulation, to the cellular stress response or any other genes related to protein damage and degradation were observed. Although a small number of studies have examined gene expression in fish

larvae in response to ocean acidification, these studies have been limited to candidate genes related to stress response [36] or acid-base regulation [12]. To our knowledge, this is the first study that addresses the effect of OA on the whole transcriptome of marine fish larvae.

Based on the observed gene expression patterns at 6 and 13 dph, Atlantic cod larvae appear to be unable to neither perceive nor to react to the increased pCO_2 by altering gene transcription patterns. Given that we assessed 8 biological replicates per treatment and age, our applied methodology should have reasonable statistical power to detect subtle expression differences caused by the ocean acidification treatment. Additionally, the fold changes of the few identified differently expressed (DE) genes are modest, ranging from $\log_2$ fold changes of -0.51 to 0.54 with the single exception of Annexin A5 with a $\log_2$ fold change of 0.91 (see SI Table. 2 and 3).

Once the larvae were further developed (36 dph), many more differences in gene expression between the treatments were observed, namely 1413 DE genes. In addition to the number of DE genes increasing with age, the fold changes of these genes also increased, with 19 genes being up or down regulated by a factor of 2 or more, a much higher fold change level than at 6 and 13 dph (SI Table 4). The observed changes in the transcriptome do not present the magnitude of transcriptomic changes one would generally expect from a stressor that has caused such high mortality [8] and physiological changes [13]. However, fold changes of differentially expressed genes identified in other studies far exceed the observed fold changes in this study. Fold changes can differ by an order of magnitude, when comparing the transcriptomic reaction to hypoxia and cold stress in zebrafish larvae [37]. Furthermore, the reaction in tissues of adult fish generally results in much higher fold changes, e.g. to temperature in rainbow fish [38] and salinity in sea bass [39], particularly in genes related to the cellular stress response. This indicates that cod larvae are either not able to produce any larger transcriptomic response to ocean acidification during early larval stages, or the regulation capacity is only limited to certain organs or developmental stages and is not detected by the applied sampling protocol (e.g. dilution effect through whole body homogenate) and life stages chosen for analysis. The presence of phenotypic responses with no transcriptomic responses has previously been observed in *Mytilus* larvae, under predicted ocean acidification, with larvae showing different growth patterns but no differences in global gene expression [40].

Proteome analysis reported only small effects of increased pCO_2 on the proteome of fish [41,42], however these analyses lack the high sensitivity/resolution of RNA-Seq studies. The proteome for acid base regulation in all organs of juvenile halibut has already been developed and varies only in 2 to 6 proteins between different tissues and CO_2 treatments [41]. Similarly, the proteome of whole herring larvae [42] uncovered only a few proteins that were present at different concentrations with low fold changes in response to high CO_2 levels.

The lack of DE genes related to the cellular stress response in 6 and 13 dph larvae suggests that, on a whole organism scale, the predicted end-of-century pCO_2 level does not trigger an initiation of the CSR in very early larval stages of Atlantic cod. The observed differential expression of CSR genes in 36 dph larvae would suggest that at this stage predicted ocean acidification does exert a sufficiently strong stressor on the cells to cause increased transcription of CSR related genes compared to ambient pCO_2

levels even though this occurs only in a relative low number of genes (14/185 annotation). Similar to the general transcriptome patterns the fold changes of DE genes were low. Heat shock protein 70 (HSP 70), one of the best characterized candidate gene in stress response to environmental change, had a fold change of only 1.68. This value (Figure 4 and SI Table 5) is much lower than in other fish studies assessing the effects of long term imposed stressors such as salinity, oxygen and temperature, where fold changes range from 2.5 to 372 FC [38,43,44]. Remarkably, in these studies, differences in diet can potentially cause larger stress responses (higher fold changes of CSR) in larval cod [45] than increased pCO_2.

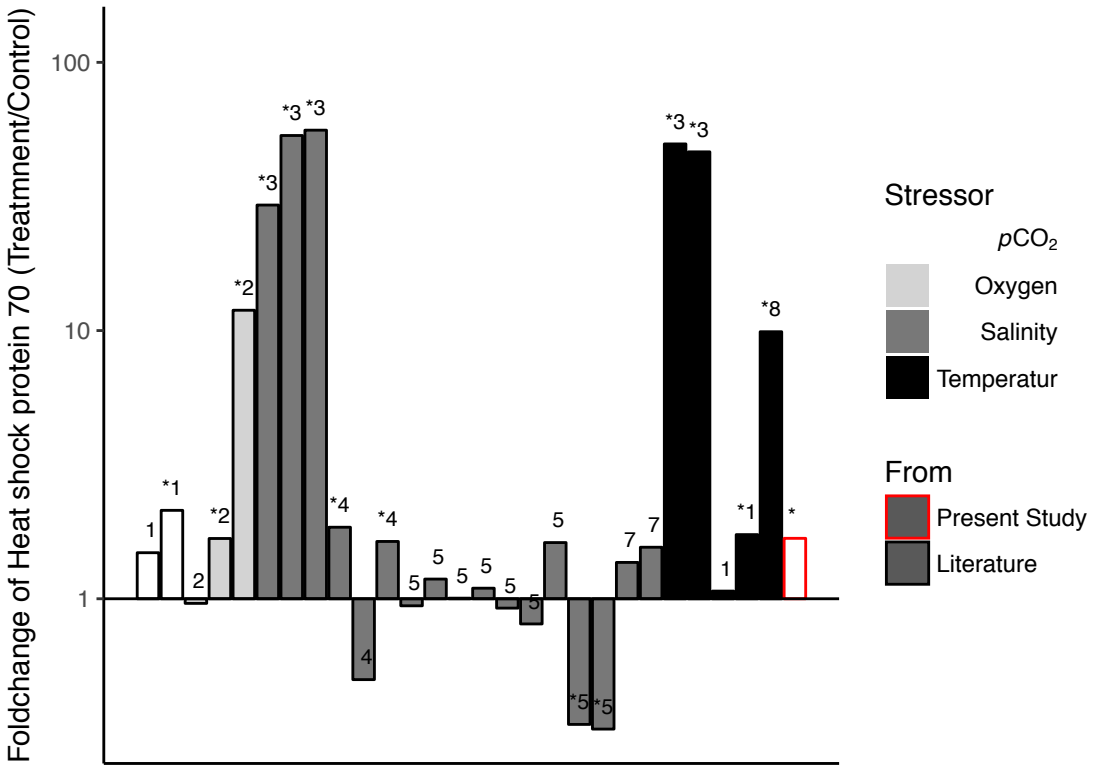

Figure 4: Heat shock protein 70 (HSP70) fold changes of fish in response to chronic exposure to abiotic environmental stressors, * marks significant differences between treatments, numbers show reference numbers in SI Table 5. For further information see SI Table 5

Previous studies have identified a variable stress response in larval fish exposed to increased pCO_2. When investigating the expression patterns of HSP70 in whole fry (8 days post fertilization) of big head carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*) in response to extreme pCO_2 levels (43000 µtam) in freshwater, only larvae from the latter species showed an upregulation in HSP70 [36]. Nevertheless, such pCO_2 levels are not possible in the most parts of the oceans. Further, the stress response to predicted future pCO_2 levels was assessed in the larvae of a tropical flatfish [46]. Protein levels of HSP70 in whole Senegalese sole (*Solea senegalensis*) larvae incubated and reared in ambient and high

pCO_2 (~1600 µatm) levels differed only in 30 day old larvae [46]. They were significantly higher in the ocean acidification treatment compared to the control, while protein levels did not differ in 10 dph larvae. This presents a similar pattern to the results of this study, indicating that only later larval stages present a measurable stress response to increased pCO_2 when analysing whole body homogenates.

None of the candidate genes linked to acid base regulation was differentially expressed in the 6 and 13 dph larvae. The use of whole larvae tissue homogenates instead of gill tissues could be questioned as suboptimal when addressing expression patterns in ion and acid-base regulation and causing potential signals to be lost in dilution, e.g. the response might be limited to an organ or to part of it and not detectable when addressing a whole-body transcriptome. The applied approach, however, allowed for an assessment of the full transcriptome profile at the individual level vs. using a single tissue. This would be less informative and thus give us information on how that specific tissue is responding to the treatment, and not the whole organism. The skin, solely responsible for acid-base regulation prior to gill formation, is impossible to separate from other tissues for single tissue transcriptome profiling in these early larval stages. No differences in the number of Na^+/K^+ ATPase rich ionocyts (chloride cells)were identified on the yolk sac epithelium of newly hatched cod larvae, important for acid-base base regulation, either [9]. All indications support the hypothesis that early stage cod larvae cannot or do not undergo changes in the transcriptome in response to predicted levels of ocean acidification.

Similar to genes related to the stress response, only 36 dph larvae showed DE genes related to acid-base regulation. Namely Carbonic anhydrase (CA), a zinc metalloenyzme, which catalyses the reaction $CO_2+H_2O \leftrightarrow H^+ + HCO_3^-$ and is crucial for acid-base regulation and CO_2 excretion in adult fish was differentially expressed. It is present in a number of isoforms with differences in properties, sequences, and tissue distribution [21,47]. The CA isoform identified to be differentially expressed in this study is CA4, a form which is known to be active extracellularly and has a large number of variants in teleost fish [21]. In a previous study, no difference in transcript abundance of any CA4 isoforms was identified in the anterior intestine of adult cod exposed to increased pCO_2 [29]. Tseng et al.[12] also identified a downregulation in CA (namely CA2 and CA15) in embryos of medaka under increased pCO_2. But no such differences were identified between hatchlings from a control and a 1200 µatm treatment. Only CA15 transcript abundances at very high pCO_2 (4000 µatm) differed compared to controls. As that study [12] assessed only selected candidate genes, the expression of CA4 was not addressed. In the present study, CA2 was not found to be DE, while CA15 is not present in the annotation. CA4 may also be involved in stimulating the activity of Na^+/HCO_3^- co-transporter 1 (NBC1, SLC4A4) [48], an important mechanism for HCO_3^- transport over the basolateral membrane [22].

The expression patterns of ammonia Rh transporters, suggested to be important in CO_2 transport across membranes [49], differ from findings in the previously cited study on marine medaka [12]. Rhcg did not differ, while Rhcg2, a close relative, was downregulated in 36 dph cod larvae. Further medaka hatchlings reacted to increased pCO_2 by upregulation of Rhcg. On the other hand, an upregulation of Rhag was observed in the same experiment [12], while the same gene was not differentially expressed in the present study. Additionally, the changes of Rhbg in this study were not identified in medaka hatchlings but only in their embryos, in which it was downregulated [12]. These differences in transcriptomic response to

ocean acidification between species and their different life stages (reviewed in [22]) could be part of the explanation why species responded in physiologically different ways to increased pCO_2.

Interestingly, insulin induced gene 1 protein (Insig 1), a gene that has been associated with hepatic steatosis in zebrafish [50,51], showed one of the largest fold change among the DE genes in the 36 dph larvae (Table 4). Hepatic steatosis or fatty liver syndrome, characterized by the accumulation of lipids in vacuoles in liver cells, could be connected to the observed tissue damages in the liver of these cod larvae under simulated ocean acidification to the molecular phenotype [13,19]. The upregulated gene col27a1b Collagen alpha-1(XXVII) chain B in 13 dph larvae (SI Table 3) is associated with calcification of cartilage into bone and particularly within the notochord [52], and potentially related to the increased number of ossified vertebrae observed under ocean acidification in the same experiment [13].

Both the results of the GO enrichment analysis and the general dynamics of DE over time suggest that the observed gene expression differences in 36 dph old larvae are driven by accelerated development, which in turn is driven by the pCO_2 treatment. This is supported by the changes in dry weight between the treatments, which increases over time: 5 and 15 dph old larvae vary only slightly, while 36 dph old larvae almost lack an overlap between the different pCO_2 levels, as clearly visible in figure 1. Possibly, larvae from ambient treatments are still in the earlier isometric growth phase at 36 dph, while larvae from the high pCO_2 treatments have entered the later allometric growth phase and undergone the related shifts in metabolic scaling [53]. Additional support was provided by a group of DE genes of relative high fold changes, known to be expressed in highly variable levels along the developmental stages of *Danio rerio* [50,54]. Protein fosB, Krueppel-like factor 4, Cyclicl AMP-dependent transcription factor ATF-3, Pleckstrin homology like domain family A member 2, Amphiregulin and Myosin heavy chain (fast skeletal muscle) (SI Table 5) are all expressed at variable levels along the larval development in zebra fish and can be assumed to follow similar patterns in other teleosts. However, it is unclear whether the developmental changes are caused by direct effects of OA on growth and ossification or if the observed differences are due to the strong mortality in the early ages potentially selecting for fast growth or combination of both with related trade-offs [13]. Furthermore, developing larvae might not be able to react at all to abiotic stressors such as pCO_2 as they are in a constant state of change. For example, with newly forming organs, they have no excess resources for a stress response leading to high mortality rates, with only well provisioned larvae being able to mobilize a response to survive critical developmental stages.

In conclusion, gene expression patterns in combination with the high mortality [8] and histological tissue changes [13] from the same experiment suggest that the predicted end-of-century ocean acidification is not detected by cod larvae as a stressor on a whole organism scale. This is also supported by target gene data related to the cellular stress response (CSR, [34]). It thus seems to constitute a "stealth stressor" that does not trigger classical stress responses but still results in tissue damages and increased mortality. This is further supported by the lack of transcriptomic signs of protein damage. Possibly, larvae that fail to keep homeostasis in terms of gene expression show rapid deterioration of gene expression patterns followed by rapid death. If so, these individuals with their deteriorated gene expression patterns would be missed by our sampling, while they would contribute to enhanced mortality. To fully understand why no cellular stress response or general transcriptomic response is present, more research is needed into

both the underlying physiology as well as into transcriptome and protein analysis. Emphasis should be placed on investigating individual tissues at different developmental stages to assess in higher resolution and pinpoint stages of high vulnerability.

Methods

Experimental setup

Adult cod were caught in the Barents Sea (70°15'N, 19°00'E) in March of 2014 and transported to the National Centre for Aquaculture, Kvaløya, Norway, where they were transferred into 25m^2 spawning tanks equipped with a flow through of sea water from the fjord of ambient temperature and salinity. Photoperiod was matched to local sunrise and sunset to induce spawning; oxygen saturation, pH, salinity and water temperatures were monitored daily. Once the fish started to spawn, all floating eggs in the spawning tank were collected using a mesh bag behind the surface skimmer. The volume of collected eggs was divided into two equal portions and moved into 28 L flow-through incubators. Half of the egg incubators were adjusted to ambient (503 ± 89 µatm) and the other half to end-of-century (1179 ± 87 µatm) pCO_2 concentration.

Two days after > 50% of larvae had hatched, 11 000 larvae were transferred into each of six 190 L rearing tanks, three replicates of each pCO_2 treatment. All larvae were fed using a reduced aquaculture feeding regime for commercial fry production (termed low food treatment in [8,13]), starting with green water addition (*Nannochloropsis*) followed by enriched *Brachionus* and later *Artemia* nauplii (See "Low food" in [13] Table 1). The water temperature for the rearing tanks was initially kept at 6°C but was increased to 10°C after day 6, while the photoperiod was kept constant throughout the experiment (24 h light). For larval weight analysis, 12 larvae from each replicate were sampled at day 5, 15 and 36 days-post-hatch (dph), euthanized using MS-222 (Tricaine methanesulfonate) and frozen in treatment water at -20°C. Single larvae for transcriptome analysis were sampled randomly from all tanks at 6, 13 and 36 dph, immediately euthanized using MS-222 and submersed in RNA-later®, placed at 8°C for 24 h and subsequently stored in –78 °C until RNA isolation.

The pCO_2 concentrations in both treatments were continuously monitored using pH sensors in the incubators, spawning and rearing tanks connected to a computer system (Aquastar, IKS Computer systems, Karlsbad, Germany). Increased pCO_2 concentrations in the water for the end-of-century treatment were achieved by aerating the header tanks with CO_2, which ensured equal and constant pCO_2 concentrations in all replicates of both ambient and high pCO_2 treatments. The absorption of CO_2 into the header tanks was regulated by a magnetic valve that controlled the aeration with CO_2 by the IKS system through pH-sensors in the outflow of the header tank. Further, temperature, salinity and pH were measured daily with a hand held multi probe (WTW pH/Cond 340i/3320). Additionally, the pCO_2 levels in the tanks and incubators were checked weekly from calculated pCO_2 using total dissolved inorganic carbon and total alkalinity and the chemical speciation calculation program CO2SYS [55], in accordance to the Best practice guide [56]. The mean pCO_2 values and standard deviation for the ambient and the high pCO_2 treatment were 503 ± 89 and 1179 ± 87 µatm, respectively, corresponding to an *in*

situ pH of 8.00 and 7.68, respectively. For more detailed information please consult the Supplementary material of [8].

Ethics Statement

This study was carried out at NOFIMA's Centre for Marine Aquaculture, Kvaløya, Norway applying methods and protocols approved by the National Regulatory Committee on the Ethics of Animal Experiments, Norway under the permit number: FOTS id 6382 and in strict accordance to the relevant regulations and guidelines. All possible actions were taken to reduce animals suffering and stress during handling and sampling.

Larval dry weight

Vials containing larvae for dry weight analysis were thawed on ice; the individual larvae were inspected for completeness under a stereomicroscope, rinsed in distilled water and placed in individual vials. Larvae were freeze dried for 16 h before being weighed to the closest 0.1 µg. Damaged and incomplete larvae were excluded from the analysis. The statistical analysis was performed in R version 3.3.2 [57], using R studio [58] and a linear mixed model (R-package lme4 [59]). Larval treatment as a fixed factor and tank as a random factor was applied for each of the 5 and 15-day old age groups. The data for 36 dph were analysed and presented in reference [13].

RNA isolation and sample preparation

We chose to investigate the global transcriptome of homogenates of whole, single cod larvae at several time points, instead of the often-used gill tissue, as organ development is constantly changing in these life stages and gills are not developed at hatch. Larvae preserved in RNALater® were thawed on ice, dapped dry on paper tissue and wet weight measured to the closest 0.001 mg. RNA from individual larvae was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) with a modified protocol implementing on-column DNase digestion steps (RNase-Free DNase Set, Qiagen, Hilden, Germany). Briefly, the whole larva was placed in the lysis solution immediately after thawing and homogenized using a TissueLyserII and glass beads (Qiagen, Hilden, Germany) for 2 min at 20 Hz before 600 µl 70% EtOH was added. 700 µl of the homogenate were transferred to the spin column and centrifuged at 8000 g for 15 sec. The flow-through was discarded (as in all following steps except elution) and the remaining 500 µl of the homogenate was added to the column before the centrifugation process was repeated. A wash step was performed before the on-column DNA digestion for 15 min at room temperature. Three additional washing steps were performed as per manufacturer specification before the column was centrifuged for 2 min at maximal speed to remove all remains of the wash buffers and to be dried. The RNA was eluted from the column using 50 µl of RNase and DNase free water for 1 min at 8000xg. Purity of the RNA extract was assessed by spectrophotometry (ND-1000, ThermoFisher Scientific, Waltham, MA, United States), quantified with a broad range RNA test on a fluorimeter (Qubit2, ThermoFisher Scientific, Waltham, MA, United States) and the RNA integrity was evaluated with a Eukaryote Total RNA StdSens chip using an Experion automated electrophoresis system (Bio-Rad, Hercules, CA, United States). Samples with purity, quantity and integrity below the recommendations and guidelines of the sequencing centre were excluded from further analysis.

Sequencing

In total, 48 mRNA (8 biological replicates per treatment (ambient and high pCO_2) and 3 sampling points (6, 13 and 36 dph) cDNA libraries were constructed using the TruSeq Stranded mRNAseq Sample Prep kit (Illumina Inc., San Diego, CA, United States; 0.5 µg total RNA input). Sequencing was performed on a HiSeq4000 (Illumina Inc., San Diego, CA, United States) platform with a 150bp paired-end protocol. After quality control of the library preparations, all samples were pooled and run across 5 lanes.

After an initial round of quality control, we detected too low sequencing depth (around 20% of average) in 5 samples, which were then re-sequenced on an additional lane to supplement the previously sequenced data to satisfactory levels.

Bioinformatics and statistical data analysis

The sequencing adaptors were removed using Trimmomatic Version 0.36 [60]. Of the resulting data, all unpaired reads were discarded and only paired reads were used for further analysis. The quality of the remaining reads was assessed with FastQC [61] and MultiQC [62]. All paired reads were compared to a transcriptome created , using getfasta of bedtools [63] based on the most recent published cod genome and its annotations [64], using Kallisto [65]. Transcript abundance data generated by Kallisto were imported into R studio [57,58] using the tximport package [66]. The differential gene expression (DE) analysis was performed using DESeq2 [67] using default settings and a FDR (false recovery rate) correction using the Benjamini-Hochberg (BH) method [68].

A gene ontology (GO) enrichment analysis was performed using GOseq [69] for all genes that were found to be significantly up or down regulated in the DE analysis at 36dph. Further, candidate genes were extracted from the existing literature, focusing on genes related to acid-base regulation [22,28] and genes related to the minimal stress response [34]. All data visualizations were created using the ggplot2 [70] or DESeq2 [67] packages in R [57].

Read counts assigned to the reference were transformed using the regularized logarithm (rlog) [67] to be used in multivariate analysis, including principal component analysis (PCA) using prcomp, to visualize clustering of samples and to assess the underlying loadings. Further an analysis of similarity (ANOSIM) in R-package vegan [71] was performed on Euclidian distances to assess differences between the age groups.

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Author Contributions

FM, MS, CC, TR planned the experiment, FM, MS, CC performed the experiment, SJ and VP supported the experiment, SJ contributed with planning of the RNA-Seq, FM performed the analysis, TB and TR supported the analysis, MC provided the carbon chemistry analysis, FM, CC and TR wrote the first draft of the manuscript, all authors contributed to the manuscript.

Competing Financial Interest

The authors declare no competing financial interests

Data Availability

RNA-Seq data has been deposited to the NCBI Gene expression omnibus (GEO) under https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108715, growth data (ages 5 and 15 dph) has been submitted to Pangea https://doi.pangaea.de/10.1594/PANGAEA.884548

DIC data is available under https://doi.pangaea.de/10.1594/PANGAEA.858615

CHAPTER 3

HUSBANDRY EFFECTS IN EXPERIMENTS ADDRESSING OCEAN ACIDIFICATION: INTERACTION WITH DIRECT EXPOSURE AND LONG-TERM PARENTAL ACCLIMATION TO INCREASED pCO_2 IN ATLANTIC COD

Husbandry effects in experiments addressing ocean acidification: interaction with direct exposure and long-term parental acclimation to increased pCO_2 in Atlantic cod

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Abstract

Simulated levels of ocean acidification have been shown to negatively impact the early life stages of several fish species, Atlantic cod amongst them. The presented study aims towards assessing the effects of long-term (5 months) parental acclimation of Atlantic cod to predicted global pCO_2 levels (~1030 μatm, IPCC RCP 8.5) compared to ambient levels (~440 µatm) on mortality, growth, condition, metabolic rates, bone ossification, lipid content and gene expression patterns in early larval stages. However, none of the effects of larval exposure to increased pCO_2 repeated in several previous studies could be found. Thus, the effect of parental acclimation to the stressor could not be investigated. But when assessing the effects of long-term husbandry of the parental generation and comparing it to previous experiments, showing a negative impact of increased pCO_2on larval fitness, a potential mediatory factor "the condition of the parental fish" could be identified.

In summary, these results reveal that larval sensitivity to ocean acidification is suggestively highly dependent on parental nutritional status in Atlantic cod.

Introduction

As a consequence of the oceanic uptake of the continuous increase in atmospheric carbon dioxide (CO_2) concentration since the onset of the industrial revolution, the ocean's carbonate chemistry has shifted to a less basic state with decreased pH and carbonate ion concentrations (Caldeira & Wickett, 2003; Doney et al., 2009). This change, known as ocean acidification (OA), has been shown to impact many marine organisms and may be a major threat to global biodiversity (Brierley & Kingsford, 2009; Kroeker et al., 2013; Wittmann & Pörtner, 2013).

While the predicted increase in ocean pCO_2 until the end of the century (IPCC, 2013) may have many detrimental effects, particular on calcifying organisms (Orr et al., 2005), fishes have long been considered highly capable acid-base regulator and thus being robust to pCO_2 levels far exceeding predicted levels (Ishimatsu et al., 2008). Yet, various studies have found that many species' early life stages, such as eggs and larvae, appear to be highly sensitive to increased pCO_2 levels. Effects of predicted ocean acidification on fish larvae include increased mortality (Stiasny et al., 2016), changes in growth (Baumann et al., 2012) and development patterns (Tseng et al., 2013), as well as changed otolith (Maneja et al., 2013a) and bone development (Pimentel et al., 2014, Stiansy et al., submitted a) and organ damages (Frommel, Maneja, et al., 2012, Chambers et al. 2014, Stiasny et al., submitted a, submitted b). Although the lack of functional gills in the early life stages is similar in most examined species, these results are neither universally applicable to the early life stages of all marine fish species, nor are all investigated traits affected for that matter. Some studies did not find any or only limited effects of increased pCO_2 on eggs and larvae (Franke & Clemmesen, 2011; Munday et al., 2011; Frommel et al., 2013; Hurst et al., 2013). Thus, while the underlying physiological responses and pathways of adult fish responding to increased pCO_2 are rather well understood (reviewed in Heuer & Grosell, 2014), only limited information is available for early life stages (Melzner et al., 2009).

Atlantic Cod (*Gadus morhua*) is one of the economically and ecologically most important species in the Northern Atlantic and adjacent seas. With the exception of behavioral traits of larvae of Norwegian coastal cod (Maneja et al., 2013b) and eggs and newly hatched larvae of the Eastern Baltic Sea cod (Frommel et al., 2013), its early life stages have been shown to be generally negatively impacted by to predicted changes in carbonate chemistry (Frommel et al., 2012; Maneja et al., 2013a; Stiasny et al., 2016; Dahlke et al., 2017). It has been hypothesized that this tolerance could be an adaptation to the low oxygen concentrations and already high CO_2 concentrations often encountered in that part of the Eastern Baltic Sea (Frommel et al., 2013). Eggs and larvae of Northeastern Arctic (NEAC), Norwegian coastal (NCC) and western Baltic Sea cod have been shown to be highly susceptible to direct exposure to predicted end-of-the century pCO_2 in numerous traits. Observations range from decreased hatching success (Dahlke et al., 2017), increased larval mortality (Stiasny et al., 2016), changed growth and developmental patterns as well as altered biochemical traits, lipid content, bone ossification, gill development and tissue damages (Stiasny et al., submitted a; Frommel et al., 2012) and otolith development (Maneja et al., 2013a). These observed negative effects on survival are leading to a projected decrease in recruitment NEAC population (Stiasny et al., 2016; Koenigstein et al., 2017).

In the field of ocean acidification research the potential for an evolutionary response to predicted pCO_2 is being increasingly addressed (Sunday et al., 2014; Torda et al., 2017). So far, only very few studies on fish have addressed the potential benefit of transgenerational acclimation effects under predicted ocean acidification. Miller et al (2012) and Allan et al (2014) report on mediation of altered metabolism and behaviour, respectively, caused by increased pCO_2 in the coral reef fish *Amphiprion melanopus* if the parental generation had already been exposed to the stressor. Welch et al (2014) on the other hand, could not find any mediation of changed behaviour after transgenerational acclimation in *Acanthochromis polyacanthus*. So far, the only studies addressing this matter in temperate fish, were conducted on stickleback (*Gasterosteus aculeatus*) (Schade et al., 2014) and Atlantic Cod (Stiasny et al., submitted b). In both cases, the parental acclimation seems to aggravate the effects of increased pCO_2 under certain conditions. The study on larval cod originating from aquaculture stocks found similar vulnerability in mortality and tissue damage, but no effect on growth (Stiasny et al., submitted b).

Most studies so far focused on the effect of a rapid and short-term exposure of ocean acidification on a single generation (Murray et al., 2017), however in natural systems organisms are gradually exposed to the increase of pCO_2 and have the chance to acclimate and adapt over generations. Results may differ between short and long-term exposure studies and in turn lead to very different assessments and predictions of a species capability to exist in the future (Miller et al., 2012; Salinas & Munch, 2012).

The present study attempts to assess the effects of long term parental acclimation (28 weeks) to ambient (~ 420 µatm) and predicted pCO_2 (~ 1090 µatm) on the offspring fitness in ambient and predicted end-of-century pCO_2 levels in NEAC, investigating mortality, growth, metabolic rate, biochemical condition (RNA/DNA), lipid content, bone ossification, gill development and gene expression. While a previous study assessed short term acclimation (six weeks) on an aquaculture stock (Stiasny et al.), no data on long-term transgenerational acclimation are available for economically important wild stocks.

Material and Methods

Experimental set-up

Atlantic cod form the Barents Sea were caught in October 2014 at 70°15'N, 19°00'E and transported to Nofima's Centre for Marine Aquaculture in Tromsø, Norway, where they were distributed evenly between two $25m^3$ spawning tanks according to sex and size. The predicted pCO_2 treatment (IPCC, 2013) global average, scenario RCP 8.5, ~1060 ppm) was initiated by adding CO_2(g) to the header tank starting on October 29th, 2014. The set pCO_2 concentration was maintained by using an IKS Aquastar Controller unit adjusting the dosage of CO_2 released into the header tank as a response to pH probe measurements in the holding tank. No CO_2 was added to the header tank of the control treatment. All header tanks were supplied with filtered sea water from the adjacent fjord. Water temperature, salinity and light-dark cycle were kept at ambient fjord levels and were adjusted weekly, to allow for natural maturation of gonads and to induce spawning. Oxygen, temperature and pH were monitored continuously by the IKS system and were further temperature and pH were measured daily for verification using a handheld

probe (WTW pH/Cond 340i/3320). For all life stages (parents, eggs, larvae) the carbonate chemistry was manipulated by adding CO_2 into the header tanks of a flow through system. The adult cod were fed three times per week to satiation with frozen capelin (*Mallotus villosus*). After 150 days of parental acclimation to each pCO_2 treatment fertilized eggs were either collected in a mesh bag behind the surface skimmer of the spawning tanks or through strip spawning of eggs and sperm and in vitro fertilization. Fertilized eggs produced with both methods were divided equally between two treatments and moved into incubators (20 liter) supplied with either ambient or high pCO_2 water at ambient fjord temperature (4.93°C) and salinity (33.7 psu), resulting in four different treatment combinations: Parents ambient – eggs/larvae ambient (AA), Parents ambient – eggs/larvae high CO_2 (AC), parents high pCO_2- eggs/larvae ambient (CA) and Parents high pCO_2- eggs/larvae high pCO_2 (CC). Main hatch occurred after 22 days corresponding to 105 degree days. On the next day (0dph) 9500 larvae from the corresponding treatment were transferred into each of the seven 190 L rearing tanks (total 28 tanks). As for the parental generation and eggs in incubators temperature and pH were checked daily using a handheld WTW pH/Cond 340i/3320 probe. The light regime was 24 h light and water temperatures were kept at ambient (~5.8 °C) until 6 dph followed by a gradual and simultaneous increase in all tanks to 10 °C at 10 dph. Larvae were fed according to a reduced aquaculture feeding protocol, previously used to assess the effects of ocean acidification on cod larvae (Stiasny et al., 2016, Stiasny et al., submitted a, Mittermayer et al., submitted), starting with the green algae *Nannochloropsis sp.* followed by rotifers (*Brachionus sp.*) and *Artemia sp.* nauplii (see Stiasny et al., submitted a, Table 1). The experiment was terminated after 35 days, prior to the larvae being weaned off live feed on to dry feed according to the applied larval rearing protocols.

Carbonate chemistry

Water samples in spawning and larval tanks as well as in the egg incubators were sampled throughout the experiment to assess the carbonate chemistry by the determination of C_T (total dissolved inorganic carbon) and A_T (total alkalinity) (Dickson 2007). C_T was determined by gas extraction and coloumetric titration and photometric detection using a Versatile Instrument for the Determination of Titration carbonate (VINDTA 3C, Marianda, Germany). A_T was measured using potentiometric titration with 0.1 N hydrochloric acid using a Versatile Instrument for the Determination of Titration Alkalinity (VINDTA 3C, Marianda, Germany). Certified Reference Materials (CRM, provided by A. G. Dickson, Scripps Institution of Oceanography, USA) was used to ensure the accuracy of measurements, both C_T and A_T were well within acceptable limits (±1 µmol kg^{-1} and ±2 µmol kg^{-1}, respectively. C_T, A_T, salinity and temperature were used to calculate all other carbonate system parameters using a CO_2-chemical speciation model (CO2SYS program(Pierrot et al., 2006)). For more detailed information on carbonate chemistry analysis methodology please consult the Supplementary information of Stiasny *et al.*(2016)

Condition of parental fish and egg sizes

To assess the long-term effects of exposure of adult cod to high pCO_2 in comparison to ambient levels several condition measurements from each sex and treatment were taken at three time points (early December, early February and mid-March) during the incubation phase as well as at the end of the experiment (after 30 weeks of parental exposure). For the intermediate and final sampling, fish were

caught with a landing net and were euthanatized by using MS222 overdose (200mg/L) (Tricaine Methanesulfonate TMS, Metacaine, Finquel) and fish length, fish weight and liver and gonadal weight were obtained. Hepato- (HSI) and gonado somatic indices (GSI) were calculated. At the final sampling fish length, fish weight, HSI and GSI were analyzed separately by sex while the HSI at the earlier samplings was pooled for both sexes, due to low sample sizes.
Diameters of fertilized and viable cod eggs from the main spawning events were measured using ImageJ (Schneider et al., 2012) to assess the size of eggs produced by both parental groups.

Determining survival, growth, metabolic rate, biochemical condition and lipid content

Mortality was estimated at 16 dph as described in Stiasny et al (2016 and submitted b). In short, after evenly distributing the larvae in the tank by increasing the aeration, a tube was vertically inserted into the tank, all larvae inside the tube were trapped by a closing mechanism and counted (and normalized to the volume of the sample), this was repeated five times to receive an average and the total number of larvae in each tank was calculated. One tank in the AA treatment was lost, when the mesh cover of the outflow was damaged. This potentially allowed for larvae being lost. This tank was excluded from the mortality analysis.

For growth measurement 10-13 larvae from each tank at 0, 13 and 34 dph were sampled at random, euthanized using MS-222 (Tricaine methanesulfonate) and immediately frozen in treatment water at -80 °C until further analysis. For measurement, the larvae were thawed on ice, rinsed in distilled water and placed on a scaled slide before being photographed under a stereomicroscope. After being photographed larvae from 13 and 34 dph were placed in individual vials and freeze dried (Christ Alpha 1-4 freeze dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany). Newly hatched larvae (0dph) were excluded from this analysis as the yolk sack was often damaged or missing, leading to incorrect weight measurements. After 16h in the freeze drier, larval weight was measured to the closes 0.1 µg (Sartorius SC2 microbalance, Sartorius AG, Göttingen, Germany). After weighing the larvae were stored at -80°C for further analysis (RNA/DNA ratio and total lipid determination). Standard length (SL)was measured on the photographs taken prior to freeze drying, using ImageJ (Schneider et al., 2012).

For estimates of specific metabolic rate ($\dot{M}O_2$), oxygen consumption rates were measured on individual Atlantic cod larva according to Thor et al. (2018) at 10 and 11 dph. One individual larva from each tank was pipetted using a cut off Pasteur plastic pipette into a 1.6 mL vial fitted with fluorescent O_2 reactive foil discs (PSt3 spots, PreSens, Regensburg, Germany). The vial was gently topped up with water from the tank and sealed with Teflon caps. After a resting period of ca. 30 min to acclimate the larvae, O_2 concentrations were measured at 0, 2.5, and 5 h using an optode O_2 system (Fibox 3, PreSens, Regensburg, Germany). For the controls, five vials were filled with water from the corresponding tanks. Oxygen consumption rate (nmol O_2 ind^{-1} d^{-1}) was calculated by subtracting the average O_2 depletion rate in the controls from the O_2 depletion rate in each of the larva containing vials (nmol O_2 L^{-1} h^{-1}) and multiplying by vial volume (L) and 24 h d^{-1}. Prior testing of the optode system at 5 °C showed a 3 min, 95 % reaction time, i.e. the period of time taken before the output reached within 5 % of the final O_2 concentration value (as estimated by exponential regression). Therefore, at every sampling event, O_2 concentration was read for 3 min., and an average of values read during the last minute was used for

calculations. $\dot{M}O_2$ (nmol O_2 µgdw^{-1} d^{-1}) were calculated by dividing by dry weight (µg) (dry weight measurements were obtained by the same method as described for the growth data).

To assess the biochemical condition of the larvae, the RNA/DNA ratios were measured from a subsample of three larvae per replicate tank using larvae which had already been freeze dried for the growth measurements. They were then analyzed using the fluorometric method by Malzahn et al.(2003). Depending on the individual larval weight, each larva was homogenized in 200, 400 or 800 µl of sodiumdodecyl sulfate Tris buffer (TE SDS 0.01%). Fluorescence measurements were obtained by using ethidium bromide. After total fluorescence was measured, RNAse was used to digest all RNA before the remaining fluorescence of the DNA was measured, allowing for RNA fluorescence to be estimated by subtracting the DNA from the total fluorescence. By using a 23S r-RNA standard (16S, 23S ribosomal RNA, Boehringer, Mannheim, Germany) for calibration, the mass of RNA was calculated, while the amount of DNA was calculated by applying a slope factor for DNA being 2.2 times higher compared to the RNA concentration slope values to account for the difference in fluorescence between the two.

A subsample of larvae from the growth analysis at 34 dph was used to determine the total lipid content. A modified Folch Method (Folch et al., 1957) was used submersing freeze dried and weighted larvae in 1.5 ml glass vials containing dichloromethane/methanol/chloroform (1:1:1 in volume), they were capped and stored for 72 h at -80 °C. Subsequently the defatted larvae were transferred to new vials and dried in a desiccator for 48 h to evaporate all remaining solvents before the defatted larvae were weight to the closest 0.1 µg (Sartorius SC2 microbalance, Sartorius AG, Göttingen, Germany). The difference in dry weight before and after defatting was calculated as lipids in % of dry weight.

Investigating skeletal ossification

For the investigation of the skeletal development, specimens from 4 randomly selected replicate tanks were fixed in 96% ethanol at 34 dph. Tissue treated in this manner conserves better than in specimens more commonly fixed in 70 % ethanol (Schnell et al., 2016). Specimens were cleared and double stained (c&s) in an acid-free c&s method, following a modified protocol of Walker & Kimmel (2007). As a first step of the clearing and staining method, specimens were stained for cartilage in 6 ml of an acid-free alcian blue staining solution corresponding to Part A of Walker & Kimmel (2007). An acid-free staining solution is advantageous as acetic acid (normally used to stain cartilage following the protocol of Taylor & Van Dyke (1985) or Dingerkus & Uhler (1977) decalcifies bone, especially when it is just about to ossify in very young larvae. After 24 hours the specimens were washed in a 70% ethanol solution to remove excessive staining and then transferred for 24 hours into a 0,5% KOH solution containing alizarin red powder and four droplets of a 3% H_2O_2 solution. In order to obtain good staining results the solution should have a dark purple coloration. The KOH/alizarin red powder/H_2O_2 solution was used to clear tissues, to stain calcified structures and to bleache melanophores. For dissection, digital documentation and storage, the specimens were transferred into a 70% glycerin solution. All specimens 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 was counted on these photographs and the notochord length (NL) digitally measured. The branchial basket of each larva was dissected out in its entirety; thereafter the third ceratobranchial was removed from the branchial basket

and photographed in order to measure the length of the ossified part of the ceratobranchial, the length of its longest gill filament as well as the area of gill filaments above the ossified part of the ceratobranchial.

Whole transcriptome analysis

For whole transcriptome analysis, eight larvae (age 34 dph) (preserved in RNAlater and stored at -20 C) from ambient pCO_2 incubated parents (AA and AC) (1 per tank plus one randomly assigned) were analyzed as described in Mittermayer et al. (submitted). Briefly, total RNA was extracted from whole individual larvae using the RNeasy Kit (Qiagen, Hilden, Germany) with a modified protocol. After assessing purity, quality and quantity 16 samples were sequenced using a HiSeq4000 (Illumina Inc., San Diego, CA, United States) platform with a 150bp Pair-end protocol after cDNA libraries were constructed using the TruSeq Stranded mRNAseq Sample Prep kit (Illumina Inc., San Diego, CA, United States) (0.5 µg total RNA input). The samples were run together with samples from Mittermayer et al. (submitted) across 6 lanes. The resulting sequences were trimmed (Bolger et al., 2014) and after quality control (Andrews, 2010) (Ewels et al., 2016) were assigned to the annotations of the most recent cod genome (Tørresen et al., 2017) using Kallisto (Bray et al., 2016).

Comparison to previous cod larvae experiments

Data for comparison of larval survival in the ambient pCO_2 treatments between the different experiments and studies were obtained from deposited data to the publication (Stiasny et al., 2016) and from the authors (Stiasny et al., submitted b).

To be able to assign the experimental fish to the two populations found in Northern Norway, e.g. the Norwegian costal cod (NCC) and the migratory Northeast Artic cod, using a principal component analysis of genotype data from 48 high-differentiation SNPs. This set of 48 SNPs had been selected from a 12 k SNP chip (The Cod SNP Consortium, in preparation; but see (Berg et al., 2015) by a Bayesian analysis for differentiation outliers (Foll & Gaggiotti, 2008). This analysis was performed with a genotype data set for 24 individuals known to be NEAC and 24 individuals known to be coastal cod according to the ecotype classification of Berg et al. (2016) , as well as 527 coastal individuals from the Eastern North Atlantic south of the range of NEAC (Barth et al., 2017). Per linkage group(Tørresen et al., 2017), the two outlier SNPs with the highest degree of differentiation between NEAC and coastal individuals, according to the results of the Bayesian analysis, were included in the set of 48 SNPs. In addition, the *Pan I* locus, which is commonly used for ecotype classification in Atlantic cod (Fevolden & Pogson, 1997), was also included in this SNP set. Fin clips from a random selection of parental cod from this experiment and from parental fish caught in the Barents Sea fish for the experiment presented in Stiasny et al. (2016, sub) and Mittermayer et al (sub) were extracted using Qiagen DNeasy Single Prep Columns (Hilden, Germany). Purity and DNA quantity were estimated by measurement in a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc.). Eurofins Genomics GmbH, Ebersberg, Germany performed a KASP assays with the above mentioned 48 SNPs (on Fluidigm BIOMARK, Fluidigm Corp, San Francisco, United States) and SNPs were called (if any SNP call differed between the 2 replicated analyses the genotype was declared 0000 in order not to bias the analysis).

To compare the condition of the parental generation from this experiment to the fish used in Stiasny et al (2016 and submitted a) field data from the same time (mid-March 2014) and area (approx. 70°15'N, 19°00'E) were obtained from the International Demersal Trawl Survey in the Barents Sea (Winter Survey, Thomas de Lange Wenneck(thomas.de.lange.wenneck@hi.no)) and the "Skreitokt" (Trawl acoustic survey of NEA cod, Knut Korsbekke(knutk@hi.no)) communicated by Knut Korsbekke.

Data analysis

All statistical data analyses were performed in R (3.3.2)(R Core Team, 2014) using R-studio (Rstudio Team, 2016) and all figures were created using the ggplot2 package (Wickham, 2009) if not otherwise stated, linear and generalized linear mixed-effect models were created and analyzed using the lme4 package (Bates et al., 2015).

After data transformation, where necessary (see SI), a Student's t-test (was performed between the two parental treatments for the final sampling separated by sex. While an ANOVA with Tukey post-hoc test was performed on the HSI (sexes pooled) over time as well as to compare the HSI in the experimental groups of the present study to the 2014 field data. Egg size was compared using a Kruskal-Wallis-test and a Dunn's post-hoc test for Kurskal-Wallis-test using Benjamini-Hochberg p-value adjustment.
To assess differences in survival a two-way-ANOVA with the parental and larval treatment as factors and their interaction was performed.

After appropriate data normalization (see appropriate SI table), a linear model, with parental and larval treatment as fixed and tank as random factor, was used for analyzing the standard length of 0, 13, and 34 dph larvae and the dry weight of 13 and 34 dph larvae. The same model was applied on RNA/DNA ratios, lipid content as well as length of ossified gill arches and area of gill filaments, all normalized to notochord length.

The number of ossified vertebrae was analyzed using a generalized linear mixed-effects model with two fixed factors (parental and larval treatment) including tank as random factor and another blank random factor to account for over dispersion under poison distribution.
Specific metabolic rates were analyzed by applying a three-way ANOVA including the parental and larval CO_2 treatments and age (10 or 11 dph) as factors with their interactions.
The differential gene expression analysis was performed in R with tximport package (Soneson et al., 2015) and DESeq2 (Love et al., 2014) using default settings and a FDR correction using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995) . For more details, please refer to Material and Methods in Mittermayer et al. (submitted)
Results of the SNP analysis were visualized in a principal component analysis (PCA) using the package ADEGENET (Jombart, 2008) in R (R Core Team, 2014).

Ethics Statement

This study was carried out under the rules and guidelines of the National Regulatory Committee on the Ethics of Animal Experiments, Norway under the permits (TRANSCOD project FOTS id 6915 and ACIDCOD

project FOTS id 7346). All possible actions were taken to reduce suffering and stress of the experimental animals during handling and sampling.

Results

Carbon chemistry

The mean pCO_2 values and standard deviation for the ambient and the high pCO_2 (predicted end-of-century) treatment during the entire experiment were 439±44 and 1027±150 µatm, respectively, corresponding to an *in situ* pH of 7.90±0.04 and 7.61±0.06 respectively. pCO_2 levels over the course of the experiment (different life stages) are presented in SI table 1.

Parental treatment and eggs

After 28 weeks of treatment in ambient or high pCO_2 the adult cod showed no differences in weight, hepatosomatic index (HSI) and gonadosomatic index (GSI) neither between sexes nor in the two treatments (SI figure 1a-d, SI table. 2). Only a difference in standard length was observed between female fish from ambient and high pCO_2 treatment, with females in the high pCO_2 being slightly longer (df=30.403, t=-2.2173, p≤0.05). Further the HSI increased continuously in both treatments during the 21 weeks incubation prior to spawning and decreased during spawning (date: DF=3, F=61.686 p=≤0.001, SI table. 3) (Figure 2). Diameter of eggs produced by the two parental groups differed significantly (Kruskal-Wallis χ^2=306.97, DF=2, p≤0.001) (SI figure 2a) with eggs from high pCO_2 fish being larger.

Effects on Larvae

Mortality was assessed for 27 tanks divided over 4 treatments at 16 dph. While the larval treatment had no significant effects on mortality (figure 1a), the parental treatment was significantly different (DF= 1, F=38.806, p≤0.001) Further there was a trend in the interaction between larval and parental treatment (DF=1, F=3,440, p≤0.1) (SI table 4).

There were no significant differences between the parental or larval treatment or in the interaction of both for SL and DW at 34 and DW at 13 dph (figure 1b and 1c) (SI table 5, SI figure 2c). But SL at 13 dph was significantly different depending of larval treatment (DF=1, F=6.6863, p≤0.05) (and interaction (DF=1, F=8.5804, p≤0.01) (SI table 5, SI figure 2d). Further, SL at 0 dph showed a trend towards larger larvae from parents in high pCO_2 (DF=1, F=3.91, p≤0.1) (SI table 5, SI figure 2b).

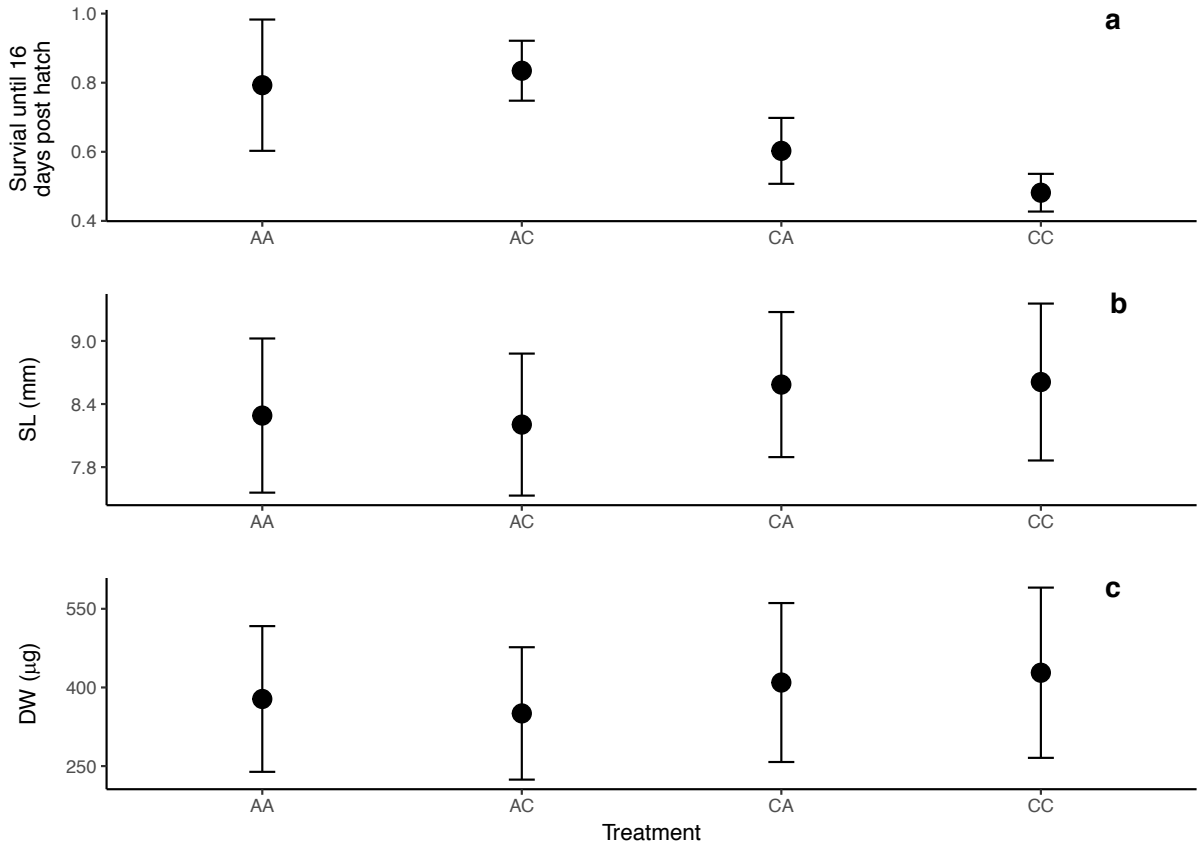

Figure 1: Effects of parental and larval treatment on larval fitness. a) Survival until 16 days post hatch, b) Standard length at 34 days post hatch and c) Dry weight at 34 days post hatch.

Respiration rates were assessed for larvae at age 10 and 11 dph (SI figure. 3a). Parental treatment had a significant effect on larval respiration (DF=1, F=10.327, p≤0.05), while larval treatment itself, the interaction of parental and larval treatment as well as the age of the larvae did not show a significant effect (SI table 6)

RNA/DNA ratio and percentage of body mass, consisting of soluble lipids, at 34 dph were not significantly impacted by neither the larval or parental treatment nor their interaction (SI figure 3b and 3c, SI table 8). The same was observed for the number of fully ossified vertebrae (SI figure 4, SI table 8) and the normalized length (to NL) of ossified gill arch, gill filament length and gill filament area (SI figure 5a-c, SI table 9).

To compare the direct effect of increased pCO_2 on larvae, 8 larval samples each from AA and AC treatment were sequenced, producing $10.4*10^9$ reads. Quality control and discard of low quality and unpaired reads resulted in an average of $64.5*10^6$ paired reads per sample, of which an average 67.7% where uniquely assigned to the reference transcriptome (SI table 10). The differential expression analysis showed no difference in gene expression between the two treatments (p_{adj}=0.05, FDR=BH). (The results are visualized as an unsupervised principle component analysis in SI figure 6 a b).

Comparison between experiments

In addition to the survival determinations in this study, seven additional responses to direct CO_2 exposure in the control treatment groups from different feeding regimes and parental treatments were comparatively analyzed (figure 2).

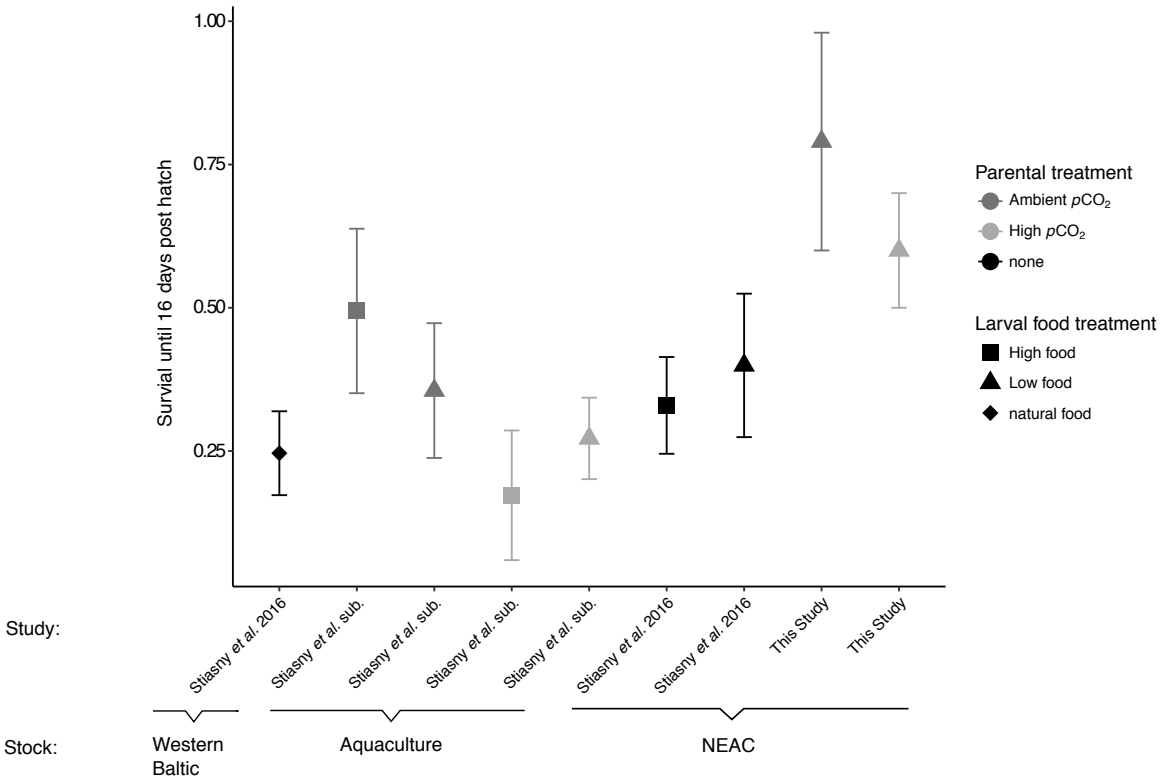

Figure 2: Survival until 16 days post hatch in larval control treatments of Atlantic cod larvae from several previous experiments, including different populations, parental acclimation and larval food treatments.

In total 14 sampled fin clips of parental fish from the present study were compared to 30 samples from the experiment described by Stiasny et al. (2016 and submitted a) and Mittermayer et al. (submitted) (from here 2014 experiment). One of these samples did not amplify and was excluded from the analysis; further 2 SNP assays did not yield any genotypes. When comparing the two groups of fish used in the 2014 experiment and the present study (figure 3) they overlapped and grouped closely with the reference population of the NEAC and were separated from the NCC population.

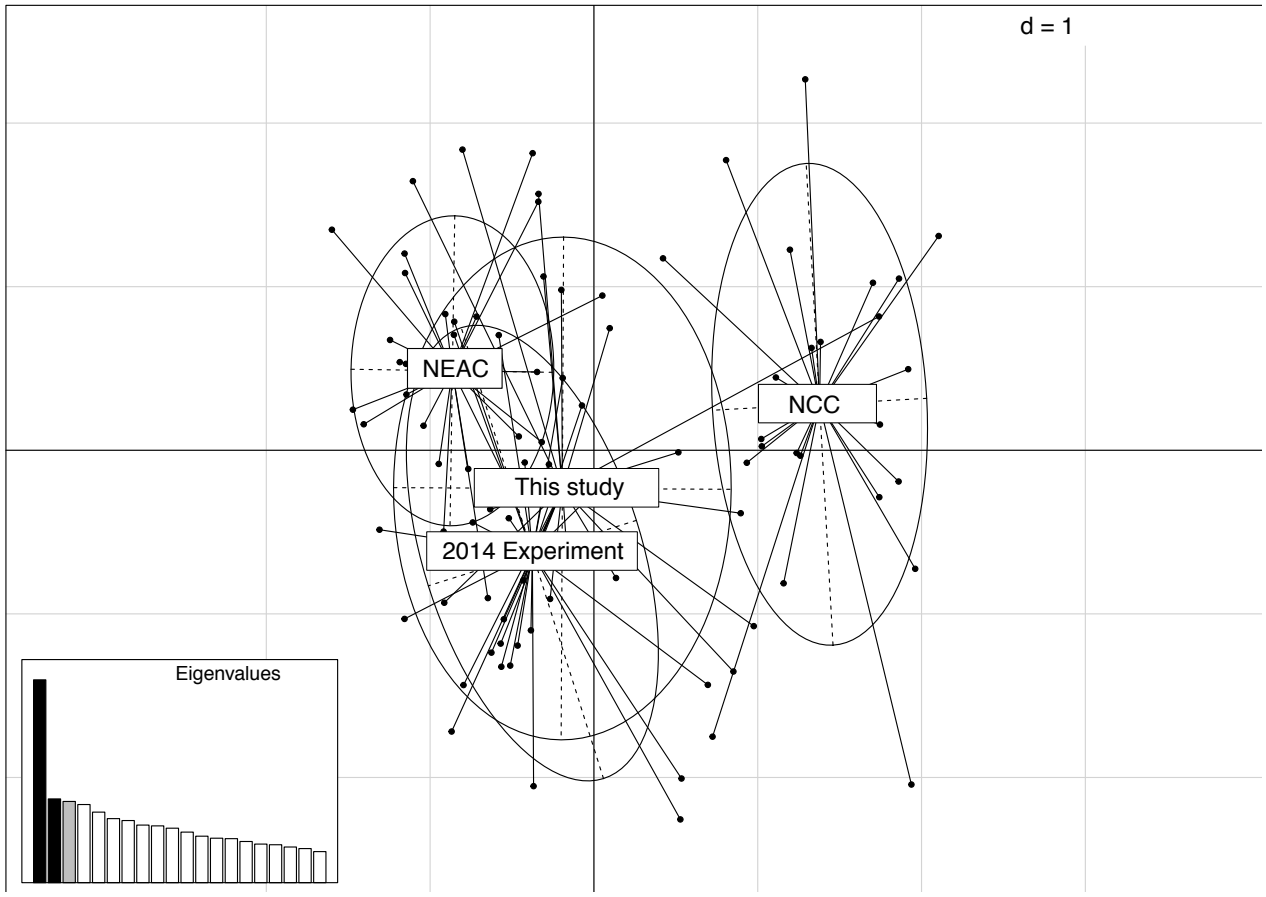

Figure 3: Principal component analysis of 48 SNP markers to assign parental fish from the present and 2014 Experiment to reference population of Northeast Arctic Cod (NEAC) and Norwegian Coastal Cod (NCC)

The acclimation over five months resulted in increased HSI by the time of spawning (mid March 2015) (figure 4) (DF=3, F=61,87, p≤0.001) Eggs produced by both parental treatments in this study were significantly larger that eggs used in the 2014 experiment (Stiasny et al., 2016) (figure. 5a, SI Table 11).

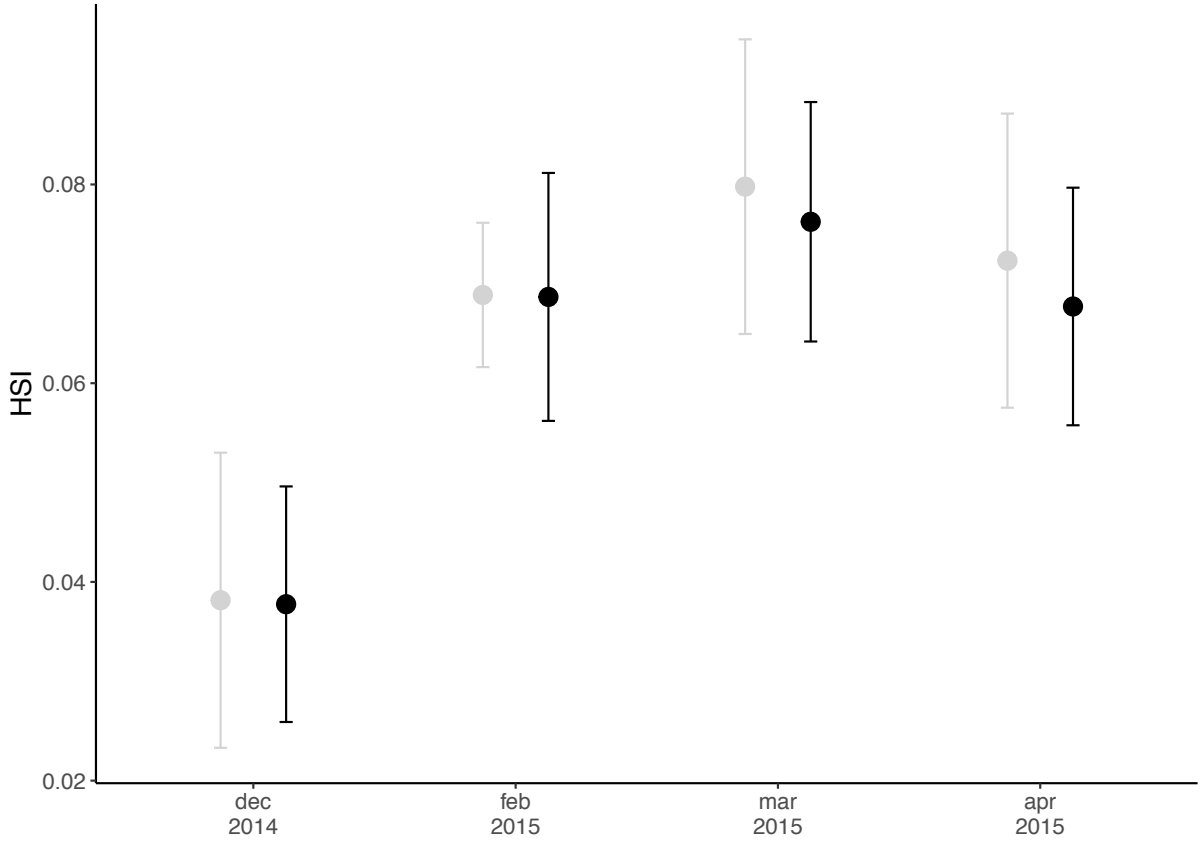

Figure 4: Parental hepatosomatic index (HSI) during the acclimation to ambient (grey) and increased (black) pCO_2, spawning occurred in mid-March

When comparing the HSI of both groups of adult fish (ambient and high pCO_2) to comparable data from wild fish of the previous spawning season (March 2014), fish taken directly from the sea had significantly lower HSI values than from both parental treatment groups in this study (DF=2, F=32.43, p≤0.001) (figure 5b, SI table 12).

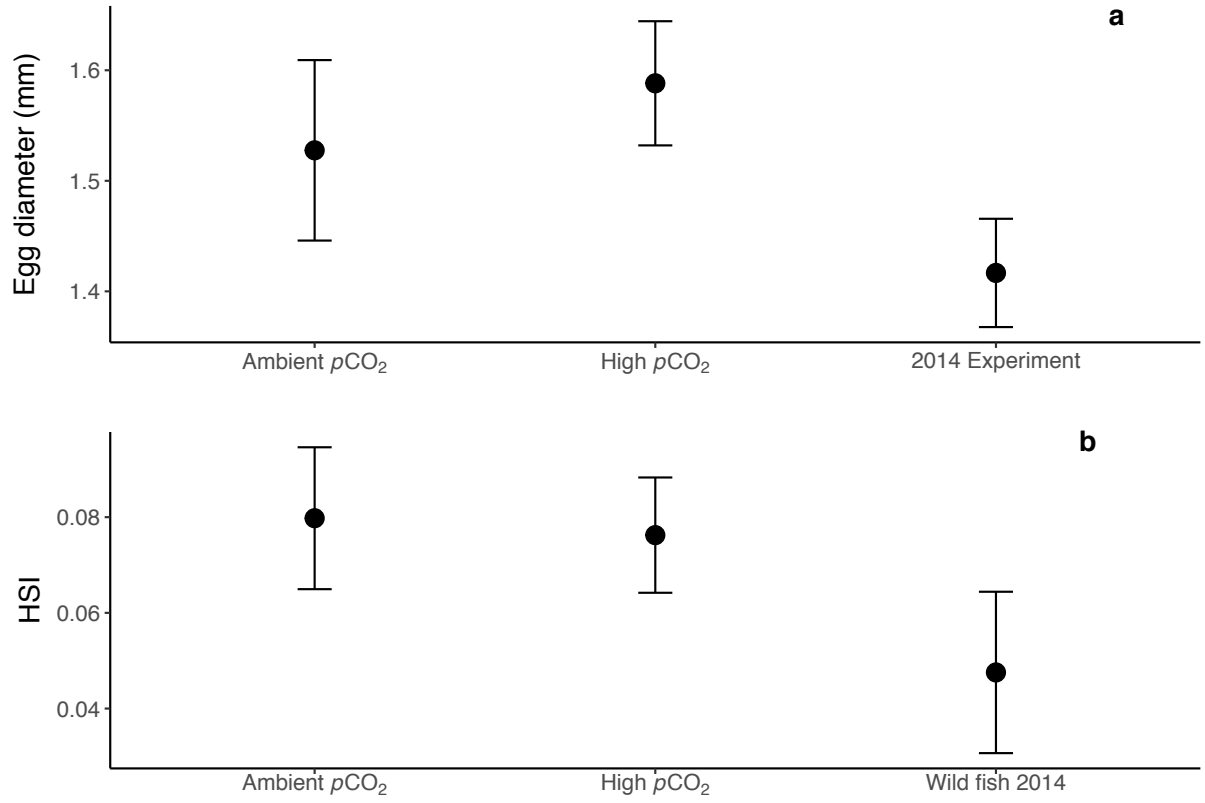

Figure 5: a) Size of eggs from ambient and high pCO_2 acclimated parents in the present study and from the 2014 experiment and b) Hepatosomatic index (HSI) of parental fish at spawning after 150 days of acclimation to ambient or high $pCO_2$and of wild fish caught in the same area and time as the parental fish used in the 2014 experiment.

Discussion

The lack of a response observed in the examined larval fitness traits to the direct exposure (larval treatment) (Figure 1, SI figure 1, 2,3,4 and 5) to increased pCO_2 did not allow for assessment of the effects of parental acclimation to the stressor. This general lack of response to the larval treatment stands in contrast to several previous studies, examining the effects of simulated ocean acidification on early life stages of cod (Frommel, Maneja, et al., 2012; Mittermayer et al., submitted; Stiasny et al., 2016;submitted a; submitted b). Further, the higher-than-expected survival in control (ambient) treatments of the present study provides an additional indicator that these results vary from earlier studies , including those performed in the same facility(figure 2) (Stiasny et al., 2016). Interestingly, the overall survival in the entire facility was very high during the time when the present study (NOFIMA, 2015). This was, in the case of the aquaculture stock produced for commercial use, attributed to continuous brood stock improvement as none of the larval rearing protocols were altered compared to previous years (personal communication Velmurugu Puvenandran & Atle Mortensen). But this

explanation is not applicable to the present study as the parental generation was wild caught. To investigate what could have caused these very unexpected results we compared the findings of this experiment to the most similar data set using data from larvae of wild caught fish exposed to a very similar pCO_2 stressor, under the same rearing protocol in the same facility under low food treatment presented in (Stiasny et al., 2016).

When comparing the 2014 experiment to the present study (table 1), it becomes evident that there is a general lack of reaction of larvae to increased pCO_2 in the present study. We had expected that, independent of the parental treatment, larvae reared in high pCO_2 would display similar reactions as observed in the 2014 experiment, e.g. as already seen for the previously repeatable outcome of mortality in response to ocean acidification of different stocks (Stiasny et al., 2016 Western Baltic compared to Wild Barents Sea compared to Stiasny et al., submitted b). However, the analyzed parameters (larval length, dry weight, ossification of vertebrae, lipid content and RNA/DNA ratio) at 34 dph in 2015 compared to 36 dph in the 2014 experiment generally reflect the state of the low food control e.g. ambient pCO_2 treatment. The lack of response becomes particularly obvious when examining the whole transcriptome analysis conducted on the larvae from ambient parents, where out of ~ 23000 gene annotations no genes were differentially expressed between the ambient and high pCO_2 larval treatments ($p_{adj} \leq 0.05$, FDR = BH). This presents a strong contrast to the 2014 experiment, where the same treatment levels resulted in 1412 differentially expressed genes ($p_{adj} \leq 0.05$, FDR = BH) (Mittermayer et al., submitted). If this differential expression pattern is due to the differential growth and developmental patterns observed in the 2014 experiment (ref Stiasny et al., submitted a) as proposed by Mittermayer *et al.* (submitted), the lack of effects on growth and developmental patterns would also result in an absence of differentially expressed genes.

Table 1: Comparisons of Mean and SD from the present study to results presented in Stiasny et al. (submitted a) and Mittermayer et al. (submitted)

Study:	Stiasny et al. submitted (low food), Mittermayer et al. submitted					this study							
Parental treatment		**none**		**none**			**Ambient pCO_2**		**Ambient pCO_2**		**High pCO_2**		**High pCO_2**	
Larval Treatment		**Ambient pCO_2**		**High pCO_2**			**Ambient pCO_2**		**High pCO_2**		**Ambient pCO_2**		**High pCO_2**	
	age (dph)	Mean	SD	Mean	SD	age (dph)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fully ossified vertebrae	35	12.1	5.8	35.8	9.9	34	13.9	13.4	16.5	13.4	21	16.7	15	11.9
DW	36	292.1	52.9	493	184.2	34	378	139	350.47	126.3	409.4	151.6	428.1	162.4
SL	36	7.80	0.48	8.95	0.84	34	8.29	0.73	8.20	0.68	8.59	0.69	8.61	0.75
% Lipids	36	22.7	2.70	18.2	3.20	34	24.89	5.70	24.83	5.35	26.92	4.48	24.93	5.18
RNA/DNA	36	1.11	0.13	1.53	0.34	34	1.17	0.23	1.25	0.27	1.22	0.28	1.34	0.34
Gene expression	36	1314 differentially expressed genes				34	0 differentially expresses genes				Not tested			

As different populations of the same species can have potentially variable responses to a stressor depending on local adaptation (Conover, 1998; Sanford & Kelly, 2011; Thor et al., 2018), it was important to ensure that we are comparing experimental results from the same population. This is particularly important in the case of Atlantic Cod, as it has been hypothesized that some populations, namely the Eastern Baltic cod (Frommel et al., 2013), have evolved a certain resistance towards increased pCO_2 levels due to the local conditions (Beldowski et al., 2010). But the Western Baltic cod reacted very similar to the Barents Sea cod in term of increased mortality to increased pCO_2(Stiasny et al., 2016). Further, a domesticated cod stock showed different reactions in terms of larval growth when exposed to increased pCO_2 (Stiasny et al. submitted b). The area where the parental fish for both, the 2014 experiment and the present study were caught were within the overlapping range of the Norwegian Coastal Cod (NCC) and the North Eastern Arctic Cod or "Skrei" (NEAC). These population are separated by behaviour e.g. spawning depth (Vikebø et al., 2007) and development (Rollefesen, 1933 as cited by Berg et al, 2016) as well as chromosomal rearrangements (Berg et al., 2016). Larvae of Atlantic cod from different population have additionally been shown to vary in growth and survival when confronted to stressors like temperature and food availability (Hutchings et al., 2007). The presented SNP data suggest that the same population, namely the NEAC (figure 3) has been used in 2014 experiment and the present study; a bias due to difference on population level can thus be eliminated.

Survival in relation to observed egg sizes between the present study and the 2014 experiment (figure 5a) matched previous findings by Paulsen et al (2009) of larger eggs allowing for a higher survival of the hatched larvae. Smaller eggs (2014 experiment) appear to have higher mortality compared the larger eggs (present study). But this pattern is evident within the present study were larger eggs (from high pCO_2 parents) exhibited lower survival compared to smaller eggs from ambient parents However, the findings between experiments are supported by the general notion that larger eggs generally result in larger larvae, but also do these larger larvae in term generally out-compete their smaller equals in unfavorable low food conditions as a higher amount of stored energy allows for longer survival (Paulsen et al., 2009). This is generally explained by a longer time until "point-of-no-return"(Yin & Blaxter, 1987) as well as more rapidly outgrowing their predators (reviewed in Sogard, 1997) (not applicable in this experiment). In addition to these two advantages, it can be hypothesized that the increased energy storage in larger eggs would allow larvae to better cope with a stressful environment. As the earliest stages of larvae (6 dph) only show limited transcriptomic response to increased pCO_2 (Mittermayer et al., submitted) it could be possible that larvae at this stage can buffer unfavorable environmental conditions not through changes in metabolism or gene expression, but through energy provision, which is dependent on the egg size.

Yet, when comparing the experimental design between 2014 experiment and this study major difference is to be found in the 150 days pre-incubation of the parental fish, during which the fish were subject to good animal husbandry for a much longer time during 2015 with *ad libitum* feeding being the most obvious influence. This feeding did improve the condition in adult cod in 2015 being reflected as energy stored in the liver, e.g. liver weight in relation to body weight (Lambert & Dutil, 1997) (hepatosomatic index)) increasing until spawning (figure 4) with a slight decrease during spawning due to loss of appetite (Kjesbu et al., 1991). Even in the wild population, increased condition in response to increased prey

(capelin) abundance has been observed for Barents Sea cod (Yaragina & Marshall, 2000). As no comparable data from the parental fish used in the 2014 experiment were available, we used data collected by the surveys of Atlantic cod caught in the same area and at the same time as the parental fish used in the 2014 experiment. As expected, regular tank feeding lead to the previously described increase in HSI which at the time of spawning exceeded the HSI observed in wild fish from the previous spawning season (used as proxy for fish in the 2014 experiment) by a factor of ~ 2 (figure 5b). Parental condition, caused by increased or altered feeding, has been shown to positively influence offspring survival (Duray et al., 1994; Donelson et al., 2008) even allowing for increasing the resilience to environmental stress (Henrotte et al., 2010). Egg size is not the only proxy for egg quality, parental condition can alter egg quality by other means than size such as maternal provisioning by alteration of yolk composition (Rainuzzo et al., 1995). Recently Snyder et al. (2018) showed that offspring sensibility to increases pCO_2 is directly connected to the provisioning of the eggs with fatty acid. We thus present the hypothesis that increased parental condition led to the production of higher quality offspring that showed higher resistance to increased pCO_2 levels. Unfortunately, the present study, in combination with other available data, could not conclusively differentiate between parental condition (HSI) and/or egg size as driving factor behind the unexpected outcome of this study. More experiments with new focus based on the present results should be conducted to disentangle the relation of parental condition and the fitness of offspring under environmental stress.

Needless to say, there are still other not investigated effects that could have caused the lack of response. Such as the stress caused by repeated emersion during catch, handling and transport just days prior to spawning in the 2014 experiment compared to the 150 days period in the present study that could have led to a decrease in gamete quality causing increased larval mortality in 2014, as has been observed in rainbow trout (Campbell et al., 1992).

Interestingly, while direct effects were absent, we found significant parental effects of increased pCO_2 on larval metabolic rate. It seems that metabolic rates may be more sensitive than the other physiological processes measured and that some factor transferred from the parents has metabolic consequences for the offspring. Metabolism is divided into one component coupled to growth and biosynthesis and another coupled to maintenance (Jobling, 1983). Although possible changes could be masked by the high variability in the data, we observed no significant decrease in growth (there was a tendency for the opposite in standard length). It therefore seems plausible that the observed lowering of metabolic rates was a result of some conditioning factor(s) carried from parents subjected to OA. Such parental effects have been observed in the anemonefish, *Amphiprion melanopus* where increased metabolic rates in juveniles caused by low pH and high temperature were reversed in the offspring (Miller et al., 2012). In the present study, it may well be that parental effects allowed decreased maintenance costs in the offspring.

Identified effects of transgenerational and parental acclimation are not universally positive, an in several cases a parental exposure to the offspring stressor caused a further decrease in offspring fitness (Uller et al., 2013; Donelson et al., 2017). This pattern has also been observed for cod. When aquaculture brood stock was exposed for six weeks to ambient and increased level of pCO_2, survival was lowest in larvae under high pCO2 from high pCO2 acclimated parents. But this pattern was only found the low food

treatment. The high food treatment of the larvae caused a positive effect in response to parental acclimation (Stiasny et al., submitted b). The observed trend in the present study of the interaction between parental and larval treatment on larval survival could then be potentially interpreted as an additive effect of parental and larval treatment being almost strong enough to redeem the positive effects caused by increased parental condition.

Independent of uncertainties on the importance and effectiveness of transgenerational acclimation and other adaptive mechanisms on the offspring fitness under climate change (Reusch, 2014; Sunday et al., 2014), the presented hypothesis of parental condition modulating the larval fitness further requires data relating to the whole food web and ecosystem changes. If ecosystems change towards a status where the parental fish are no longer able to reach a good condition, as seen by natural variation already (Yaragina & Marshall, 2000; Kjesbu et al., 2014), this might not only result in a decrease in fecundity but also a change in offspring reaction. As the effect of climate change on ecosystems in respect to fish is still relatively unknown(Sswat et al., in review; Goldenberg et al., 2017), more information needs to be gathered to confidently predict the future of marine fish populations under future global change. However, independent of the availability of data on future ecosystem state in the Barents Sea, our data indicate that large eggs, which are generally produced by larger cod, are more resistant to predicted near future pCO_2 values. This suggests that a good approach to ensure future ecological and economical viable cod stocks is to enable sufficient recruitment by retaining a large spawning stock biomass with a large spread of animal sizes and age groups.

Conclusions

The potential for mediation of adverse effect of OA on cod larvae by parental acclimation in this study could not be assessed as controls did not exhibit the well described detrimental reaction to increased pCO_2 observed in many previous studies without long-term acclimation and husbandry mechanisms of the parental generation. Based on these findings we suggest that results of direct exposure experiments of fish larvae to OA, particularly those finding the lack of effects, should be skeptically reviewed with a focus on whether the parental generation was under human care for any extended period prior to gamete collection. While a parental acclimation to laboratory conditions prior to the start of experiments is generally a good practice to reduce the variations caused by the recent history of the animals prior to capture, too good care given to the parental generation, meaning excess food, lack of predators and pathogen control could potentially lead to increased condition of parents and gamete quality when comparing to animals directly taken from the wild. This could further result in experimental outcomes that do not reflect the *in situ* conditions and potentially create false assumptions on the robustness of fish larvae in response to predicted ocean acidification. If, additionally to the gamete and offspring quality, the eggs size, as a function of maternal age and size, is the main driver behind larval fitness under increased *p*CO2 the need of retaining a spawning stock biomass with a good age structure is apparent. Further experiments are needed to disentangle these factors and add to our understanding of transgenerational effect as well as to provide quantitative results for modeling approaches.

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Author Contributions

FM, MS, SJ, CB, TR and CC planned the experiment, FM, MS, GG and CC performed the experiment, AM, CB, SJ and VP supported the experiment, MC provided carbonate chemistry analysis, NS, GG and PT provided analytic methods tools and data. SJ contributed with planning of the RNA-Seq, SJ together with JMIB, MM and PRB designed and initiated the screening of the sub-set of SNPs for further genotyping analyses, FM performed the analyses, FM and CC wrote the first draft of the manuscript, and all authors contributed to the manuscript.

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SYNTHESIS AND OUTLOOK

SYNTHESIS

During the last decade, research on the effects of ocean acidification on marine biota has expanded rapidly. A relatively new focus are the early life stages of fish, and results of partially severe physiological impairment falsified earlier notions that fish as potent regulators of ion concentration and pH should be largely unaffected by ocean acidification. Nevertheless, the scientific community remains at least partially divided regarding the question of a general vulnerability of fish embryos and larvae to increased pCO_2 levels. This dissertation set out to investigate some of the yet unanswered questions in this field, particularly the impact of ocean acidification on gene expression patterns and the effects of parental acclimation to high pCO_2 in relation to larval fitness.

The findings presented in this thesis (figure 4) are in accordance with the current state of knowledge on the effects of larval fish in response to exposure to simulated ocean acidification, namely showing both impacted and unaffected traits in cod larvae. However, the unpresidented combination of many examined phenotypic responses and next generation transcriptome sequencing allowed for a new, more comprehensive understanding of the effects of increased pCO_2 on larval physiology (chapter 1 and 2). Further, the unexpected outcome of the long-term parental acclimation experiment has led to a new hypothesis on why certain studies have not found any effects of ocean acidification on early life stages (chapter 3). This thesis clearly demonstrates the need to examine as many response variables as possible to assess whether an organism is negatively impacted by an environmental stressor.

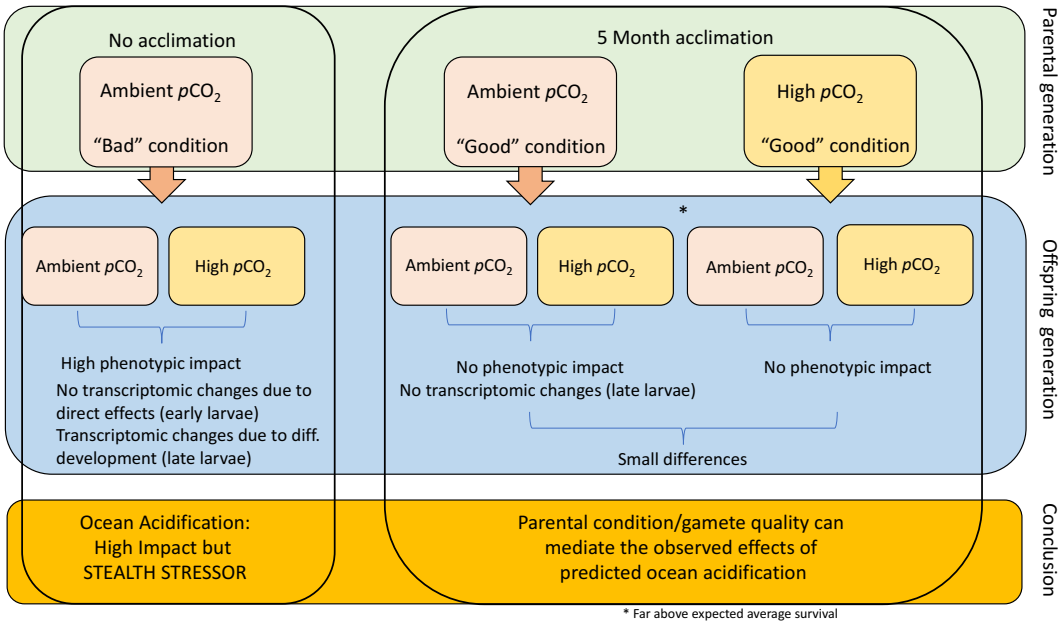

Figure 4: Schematic summary of results from chapters 1 & 2 (left vertical oval) and chapter 3 (right vertical oval). Phenotypic results include survival, growth, bone development, tissue damages, metabolic rate, RNA/DNA and lipids.

Larvae of Atlantic cod phenotypes are highly impacted by increased pCO_2 (cf Chapter 1). Growth, development and ossification are affected, additionally severe tissue damages in liver and several other organs were observed. The effect of increased pCO_2 levels on gill development was, besides the previously documented increased mortality (Stiasny et al., 2016), the only indicator that larvae under high food availability were also detrimentally affected. It would have been highly interesting to document whether or not most severely affected larvae were continually removed via natural selection (mortality) throughout development as suggested based on tissue damage, growth and lipid contents by Frommel et al. (2012). The present experiment supports the need for sampling across multiple developmental stages. If only early larvae would have been sampled, any effects of increased pCO_2 would have been missed. However, due to the applied aquaculture rearing protocols a continuation of the experiment beyond 36 days was not possible, as the weaning off life feed to dry feed usually causes a large additional mortality that could have confounded possible selection effects of ocean acidification

Larval transcription profiling led to the conclusion that early life stages of Atlantic cod were transcriptomically not impacted by increased pCO_2 levels, implying that ocean acidification represents a "stealth stressor". A "stealth stressor" being a stressor that caused a high mortality (Stiasny et al., 2016) and tissue damages (chapter 1) while at the same time did not trigger transcriptomic changes (chapter 2). This was further supported by the absence of differentially expressed genes related to the cellular stress response (Kültz, 2005), a highly conserved set of genes coding for proteins and enzymes that protect, repair and degrade macromolecules of a cell if they are damaged due the abiotic changes in the cellular environment. While the increase in otolith growth in previous experiments (Checkley et al., 2009; Maneja et al., 2013a) has been attributed to increased extracellular bicarbonate concentrations to buffer CO_2 levels, no such explanation is available for the observed increase in bone ossification. However, one of the few genes differentially expressed in the early larval stages of cod was collagen alpha-1(XXVII) chain B (col27a1b), a gene of importance in skeletal development. This presents a potential cause of the observed increased ossification under high pCO_2. Dahlke et al. (2017) observed that ionocyte morphology and concentration, important for acid-base regulation of larvae, was not affected by pCO_2 in newly hatched cod larvae. They further hypothesized that additional costs could originate from other regulatory mechanisms besides proton excretion such as defense mechanism against macro molecule damages. However, no such pattern was identifiable when investigating the transcriptome of 6 dph cod larvae (chapter 2).

The results of my transcriptome study are in accordance with the only other similar study on early juvenile Sea bass (Crespel et al., 2017), where a pattern of few differentially expressed genes was also found. But while the CO_2 treatment did not cause diverging development in sea bass, cod larvae progressively diverged in size and bone development. Such divergent development is the most likely cause of the observed difference in the late larval cod transcriptomes among treatments.

Full transgenerational acclimation, e.g. continuous exposure to the stressor for an entire life cycle, is not feasible with long lived, late maturing (5-7 years in NEAC) species such as Atlantic cod, making parental acclimation prior to gamete collection the only possible experimental approach to assess acclimation effects over 2 generations. For the NEAC population, an experiment that was to address full transgenerational acclimation would thus need to run for at least the same time (from fertilization to

fertilization). Even though short term parental acclimation has been shown to not mediate the effects of a stressor in the same degree as full transgenerational acclimation (sex ratio under warming, Donelson & Munday, 2015), the length of the parental exposure or acclimation did significantly improve the performance of the offspring.

However, it is important to state again that no clear consensus has yet been reached on the positive mediatory effect of transgenerational acclimation. Many studies on parental or transgenerational acclimation result in lack of change in offspring fitness or even document an additional negative effect of parental exposure to the stressor (reviewed in Uller et al., 2013 and Donelson et al., 2017).

Chapter 3 aimed towards estimating these effects on the fitness of Atlantic cod larvae in response to predicted levels of ocean acidification. As it turned out, two unintentional confounding factors compared to the previous studies on North East Arctic cod (Stiasny et al., 2016, Chapter 1&2) were observed: (1) an atypical, overall high survival of larvae shared in all treatments and in the aquaculture production that ran in parallel and (2) a different constitution of the parental generation. As none of the applied larval rearing protocols were altered between the experiments, the only factor that would allow for at least a partial explanation of the unexpected results is the constitution of the parental generation. The observed increase of parental condition (hepatosomatic index) during acclimation, due to husbandry in captivity, or the larger egg sizes are most likely indicator for the unexpected larval responses (mortality, growth, development, etc.). Transcriptomic analyses between larvae from ambient and high pCO_2 larval treatment, both from parents acclimated to ambient pCO_2 conditions revealed no differentially expressed genes. This absence in gene expression response and lack of indications for different development between the treatments (Chapter 3) strengthens the conclusion that the largest part of differential gene expression in older cod larvae (Chapter 2) is due to their differential development and not the direct effects of increased pCO_2.

In the light of the 2015 experiment a critical question is whether or not the general conclusion of enhanced mortality due to simulated ocean acidification holds true. In order to assess this, a meta-analysis, as a statistical tool to summarize studies, was performed. Even though the study presented in chapter 3 did not identify the previously observed significantly increased mortality in response to increased pCO_2 (Stiasny et al., 2016, submitted), the meta-analysis (for detailed methods see supplementary information) based on all available data of larval survival in response to increased pCO_2 shows a clear overall negative effect of simulated ocean acidification on Atlantic cod larvae survival (figure 5).

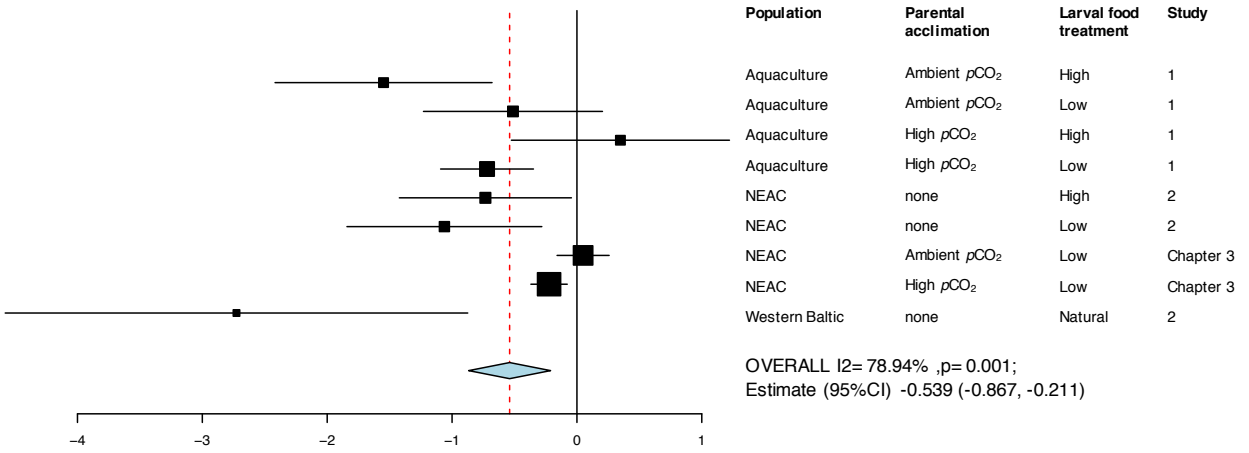

Figure 5: Results of a meta-analysis on survival of Atlantic cod larvae at 16 days-post-hatch. Data sources (1) Stiasny et al.(submitted), (2) Stiasny et al. 2016 and Chapter 3. X-axis presents the Ln response ration of larval mortality in ambient compared to high pCO_2 treatments. Each square (ln Resp. ratio) with whiskers (Var. ln. Resp. ratio) corresponds to the combination of population, parental treatment and food level (see corresponding table) while square size is the weight of each data set in meta-analysis.

Based on the growing body of publications on effects of increased pCO_2 on early life stages of fishes, it becomes evident that proposed effects of parental condition (chapter 3) have often been entirely neglected. In many cases, there is little to no information on the parental population, how long they have been kept in captivity, how they have been fed and how their own condition as well as offspring quality compares to wild populations. Particularly, when the parental generation has been held in captivity for most of their life, is part of an aquaculture-breeding program, or resulted from domesticated animals. Results concerning resilience should not be directly transferred to wild populations. As unintentional selection could have occurred, favoring robust genotypes to the stressor (Ellis et al., 2016). In general, fish from aquaculture stocks might have, in addition to the "hidden treatment" of good husbandry, been accidently exposed to transgenerational acclimation to increased pCO_2. This is caused by the accumulation of CO_2 from respiration in intensive, high density aquaculture, particular in recirculation systems resulting in pCO_2 levels far exceeding the levels projected to be reached by climate change (Ellis et al., 2016).

FUTURE PERSPECTIVES

Base on my results and the recent doctoral dissertation by Martina Stiasny (2017), it is evident that additional research on the topic of larval fish resilience and susceptibility to ocean acidification is needed, particularly in commercially important species such as cod. Knowledge gaps remain in several key areas such as ecophysiology, evolutionary ecology and epigenetics. The remaining questions include the identification of mechanisms through which increased pCO_2 levels affect the larvae physiologically

and cause increased mortality and other observed phenotypic responses. Particularly, an adjusted sampling approach, covering additional developmental stages as well as organ specific sampling, would allow for future transcriptomic and possibly proteomic studies which could yield interesting results and help to pinpoint traits that allow certain species to be resilient to increased pCO_2 while others such as Atlantic cod are so heavily impacted.

The importance of transgenerational effects and parental acclimation, a major objective of my thesis, remains partly unanswered. The absence of direct effects in larvae under direct exposure to increased pCO_2, suppose to serve as a "control" to asses the effects of parental acclimation, did not allow quantifying any possible effects of the parental treatment in combination with larval treatments exposure. As it is difficult to perform comprehensive, long-term expriments with such as late maturing and large bodied fish as cod it is important to desing the experimental set-up in a way to control for as much of the variation as possible. For instance, parental genotypes should, in ideal case, be addressed by an experimental family design to account for possible variation in offspring fitness. A future addition could even address changes in the larval microbiome under simulated ocean acidification, as potentially important for an organism's ability to cope with climate change (Torda et al., 2017). Future studies should focus on the connection between parental and larval genotype to larval fitness, when that at least some parts of a population should harbor genotypes that result in higher than average fitness under an environmental stressor (Munday et al., 2009a; Pespeni et al., 2013; Schunter et al., 2016). To address this, it would be interesting to assess the fitness of offspring from different families, most easily by investigating family dependent mortality. Initial trials have been performed in our group, but limitations in the experimental design did so far not allow for any conclusive quantification and reasons for variable fitness of cod larvae from different parents in response to simulated ocean acidification. Yet another question that should be addressed is to what degree local adaptation to hypercapnia can change the reaction norm in certain populations (Thomsen et al., 2017). While strong effects of this have been suggested for Eastern Baltic cod (Frommel et al., 2013), comprehensive common garden experiments could not only yield answers on local adaptation, but further allow to once more pinpoint traits causing resilience to simulated ocean acidification. As for investigating the mechanism underlying transgenerational plasticity, epigenetic changes in response to parental exposure must be investigated with more suitable breeding designs. However, this is due to the long generation time and large body size not feasible for Atlantic cod and should be addressed using other fishes.

Future experiments should not be confined to the initial larval stages, but continue further along all developmental stages until adulthood in order to identify possible long-term effects that could have high ecological impacts, but would remain unnoticed in short term experiments (Murray et al., 2017).

Further, the proposed effect of parental condition on offspring susceptibility needs to be examined in a comprehensive experiment, specifically designed to disentangle confounding influences of parental condition, as suggested in chapter 3, and potential family effects. Meta-analysis methods should be applied to assess whether effects of this "hidden treatment" of parental husbandry could be the cause of the highly variable findings in this research field. Results from such analyses, both experimental and from the literature, could give suggestions if the experimental set-ups and the origin of the study organisms, has an impact on how the early life stages will react to the encountered stressor. This is particularly

important as the material used in the studies ranges from wild caught larvae for behavioural trials (Munday et al., 2010), spawning of wild caught parents (Baumann et al., 2012; Stiasny et al., 2016), rearing of wild caught juveniles until spawning (Hurst et al., 2013, 2017) to using parental material from aquaculture stocks (Pimentel et al., 2014b; Frommel et al., 2016). And finally, as ocean acidification is not the only environmental change to be encountered in the near future, experiments should incorporate other abiotic factors such as hypoxia and warming to assess potential additive effects and interactions, as they might, synergistically, exacerbate effects (Przeslawski et al., 2015).

CONCLUDING REMARKS

As we live in the Anthropocene, the age characterized by human activities, ecosystems are under a rate of change unprecedented in geological history. Humankind is still fully dependent on its surrounding world and the ecosystem services it provides. This includes food security from the oceans, including many low income nations relying heavily on protein from the sea (Allison et al., 2009). It is therefore of equal importance to reduce the impact of anthropogenic activities as well as to understand how activities in the past and present will continue to impact the future oceans. A clear and coherent understanding of how the ecosystems and key species will react to climate change is important, but in order to make fully competent decision the knowledge of the underlying mechanism is paramount for our future.

In summary, we can conclude that transcription profiling and phenotypic effects under simulated ocean acidification correlated only poorly. The parental environment and condition must be taken into consideration when performing experiments, as they might have the ability to affect offspring fitness more strongly that ocean acidification by itself. Since food in the wild is always limited, and conditions for cod are deteriorating in many areas of the world (Eero et al., 2015), larvae of Northeast Arctic cod could be considered highly impacted by predicted levels of future ocean acidification.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, Felix Hans Mittermayer, dass die vorgelegte Dissertation:

Effects of Ocean Acidification on the Transcriptome of Larval Atlantic Cod and Impacts of Parental Acclimation

von mir, unter Beratung meiner Betreuer, selbständig verfasst wurde, nach Inhalt und Form meine eigene Arbeit ist und keine weiteren Quellen und Hilfsmittel als angegeben verwendet wurden.

Diese Dissertation ist unter der Einhaltung guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden und wurde weder im Rahmen eines Prüfungsverfahrens vorgelegt, noch anderswo veröffentlicht.

Zur Veröffentlichung eingereichte Manuskripte sind im Appendix unter „Author Contributions“ kenntlichgemacht.

Ich erkläre mich damit einverstanden, dass diese Dissertation an die Bibliotheken des GEOMAR Helmholtz Zentrum für Ozeanforschung und der Christian-Albrechts-Universität zu Kiel weitergeleitet wird.

Kiel, den 28 März 2018 ……

Felix Mittermayer

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M. Stiasny, M. Sswat, Michael, F. Mittermayer, I-B Falk-Petersen, N. Schnell, V. Puvanendran, A. Mortensen, TBH Reusch und C. Clemmesen, **Impacts and trade-offs of ocean acidification on growth, skeletal, and organ development of Atlantic cod larvae** (Submitted to Global Change Biology manuscript ID GCB-17-1879).

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Contributions: FM, MS, CC, TR planned the experiment, FM, MS, CC performed the experiment, SJ and PV supported the experiment, SJ contributed with planning of the RNA-Seq, FM performed the analysis, MC provided the carbon chemistry analysis, TB and TR supported the analysis, FM, CC and TR wrote the first draft of the manuscript, all authors contributed to the manuscript.

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Temming, A., Niemax, J., Zaiko, A., Siaulys, A., Törnroos, A., Clemmesen, C., Jaspers, C., Oesterwind, D., Bonsdorff, E., Mittermayer, F., Kuosa, H., Ojaveer, H., Behrens, J., Peters, J., Renz, J., Kotta, J., Dutz, J., Lehtiniemi, M., Winder, M., Setälä, O., Kotterba, P., Puntila, R. and Neuenfeldt, S. (2015) Review of environmental factors influencing distributions of selected Baltic species. BIO-C3 Deliverable, D1.1 EU Bonusproject BIO-C3, 75 pp.

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Stiasny, M., Mittermayer, F., Göttler, G., Bridges, G., Falk-Petersen, I-B., Puvanendran, V., Mortensen, A., Reusch, T. B. H. and Clemmesen, C. (submitted) Transgenerational acclimation effects to Ocean acidification on Atlantic cod larvae. Scientific Reports

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Poster presentations

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Mittermayer F., Stiasny M., Clemmesen C. and Reusch TBH. (2015) "Can Cod Cope -Epigenetic and Transgenerational Response to Ocean Acidification." 14th CeMEB Assembly, 6.-8. Oktober 2015, Tjärnö, Schweden

Mittermayer F., Stiasny M., Clemmesen C. and Reusch TBH. 2015 Transgenerational Epigenetic Effects of Ocean Acidification on Barents Sea Cod FRAM Science Days, 10.-11. November 2015, FRAM Center, Tromsø, Norway.

APPENDIX

SUPPLEMENTARY INFORMATION CHAPTER 2

Table 1: Results of a linear mixed model for dry weight of 5 and 15 dph larvae, including tank as a random factor

Larval age	Response	Fixed factor		F	DF	p-value
5 dph	Dry weight	Larval (ambient/increased)	pCO_2	6.0045	1	0.075
15 dph	Dry weight	Larval (ambient/increased)	pCO_2	2.0643	1	0.246

Table 2: All differentially expressed genes in 6 dph larvae between ambient (~503µatm) and high (~1179 µatm) pCO_2

Gene (gene name in uniprot/swissprot)	Involved in	log_2 Fold Change	L_2 FC SE	p-value	p adjusted (FDR=BH)
MEP1B Meprin A subunit beta (Homo sapiens)	Hydrolase, Metalloprotease, Protease, Inflammatory response	-0.38	0.08	1.45E-06	0.01373
Ugt2a2 UDP-glucuronosyltransferase 2A2 (Mus musculus)	Glycosyltransferase, Transferase	-0.34	0.07	8.66E-07	0.01373
ANKRD6 Ankyrin repeat domain-containing protein 6 (Homo sapiens)	Positive JNK cascade regulation, Wnt Pathway	-0.39	0.08	1.81E-06	0.01373

Table 3: All differentially expressed genes in 13 dph larvae between ambient (~503µatm) and high (~1179 µatm)

Gene (gene name in uniprot/swissprot)	Involved in	log_2 Fold Change	L_2 FC SE	p-value	p adjusted (FDR=BH)
ANXA5 Annexin A5 (Macaca fascicularis)	Blood coagulation, Calcium ion binding	0.91	0.11	3.11E-17	7.10E-13
Protein of unknown function		0.54	0.11	3.87E-07	0.00441
blm Bloom syndrome protein homolog (Xenopus laevis)	DNA-binding, DNA replication	-0.48	0.10	8.18E-07	0.00622
zpld1 Zona pellucida-like domain-containing protein 1 (X. laevis)	Membrane component	0.49	0.10	3.45E-06	0.01310
C1QL4 Complement C1q-like protein 4 (Homo sapiens)	Identical protein binding, negative regulation fat cell differentiation and fibroblast proliferation	0.51	0.11	2.95E-06	0.01310
CILP2 Cartilage intermediate layer protein 2 (Homo sapiens)	Extra cellular matrix	0.45	0.10	2.82E-06	0.01310
Nol6 Nucleolar protein 6 (Mus musculus)	RNA-binding, RNA transport	-0.51	0.11	4.40E-06	0.01434
KIAA1586 Uncharacterized protein KIAA1586 (Homo sapiens)	Ligase activity	-0.48	0.11	1.25E-05	0.03057
col27a1b Collagen alpha-1(XXVII) chain B (Danio rerio)	Calcification of cartilage, bone development	0.42	0.10	1.60E-05	0.03057
GATM Glycine amidinotransferase, mitochondrial (Gallus	amidinotransferase activity	-0.42	0.10	1.69E-05	0.03057

gallus)					
NR4A3 Nuclear receptor subfamily 4 group A member 3 (Homo sapiens)	DNA binding, Transcript regulation	-0.48	0.11	1.45E-05	0.03057
Irg1 Cis-aconitate decarboxylase (Mus musculus)	Antimicrobial, Inflammatory response	-0.43	0.10	1.88E-05	0.03057
Protein of unknown function		-0.49	0.11	1.24E-05	0.03057
comA Comitin (Dictyostelium discoideum)	Actin-binding	-0.46	0.11	1.81E-05	0.03057
SPR Sepiapterin reductase (Homo sapiens)	Oxidoreductase	-0.39	0.09	2.69E-05	0.04090
Trypsin I-P1 (Gallus gallus)	Hydrolase, Protease, Digestion	0.46	0.11	2.97E-05	0.04235

Table 4: Differentially expressed genes with L2FC ≤-1/≥1 in 36 dph larvae between ambient (~503µatm) and high (~1179 µatm)

Gene (gene name in uniprot/swissprot)		log_2 Fold Change	L_2 FC SE	p-value	p adjusted (FDR=BH)
Fosb Protein fosB (Mus musculus)	DNA binding	-2.29	0.18	1.22E-37	2.35E-33
Klf4 Krueppel-like factor 4 (M. musculus)	DNA binding, Transcription regulation	-1.79	0.18	1.26E-23	8.13E-20
ATF3 Cyclic AMP-dependent transcription factor ATF-3 (Homo sapiens)	DNA binding, Transcription regulation	-1.68	0.17	2.05E-23	9.87E-20
Ptchd3 Patched domain-containing protein 3 (M. musculus)	Membrane component	-1.54	0.22	6.74E-12	9.28E-09
lin28a Protein lin-28 homolog A (Danio rerio)	RNA binding, gene silencing	-1.33	0.20	1.49E-11	1.92E-08
AREG Amphiregulin (Homo sapiens)	Growth factor	-1.33	0.15	2.37E-19	7.63E-16
KLF2 Krueppel-like factor 2 (Homo sapiens)	DNA binding, Transcription regulation	-1.30	0.11	2.98E-34	2.87E-30
phlda2 Pleckstrin homology-like domain family A member 2 (Salmo salar)	Membrane component	-1.18	0.12	2.55E-22	9.84E-19
Fosl1 Fos-related antigen 1 (Rattus norvegicus)	DNA binding	-1.18	0.21	4.24E-08	2.21E-05
Trim39 E3 ubiquitin-protein ligase TRIM39 (M.s musculus)	Identical protein binding, Transferase	-1.17	0.22	6.95E-08	3.11E-05
Protein of unknown function		-1.13	0.17	3.55E-11	4.03E-08
PTGER4 Prostaglandin E2 receptor EP4 subtype (Homo sapiens)	Numerous functions	-1.08	0.22	1.46E-06	0.00032
Sgms2 Phosphatidylcholine:ceramide cholinephosphotransferase 2 (R. norvegicus)	Membrane component	-1.03	0.15	4.30E-12	6.92E-09
Myosin heavy chain. fast skeletal muscle (Cyprinus carpio)	Actin binding muscle protein	1.00	0.22	6.23E-06	0.00101

Protein of unknown function		1.01	0.22	6.53E-06	0.00103
ADPRH [Protein ADP-ribosylarginine] hydrolase (Homo sapiens)	Cellular protein modification	1.01	0.21	1.53E-06	0.00033
insig1 Insulin-induced gene 1 protein (Xenopus tropicalis)	Lipid and Cholesterol metabolism	1.04	0.20	3.68E-07	0.00011
ENTPD5 Ectonucleoside triphosphate diphosphohydrolase 5 (Gallus gallus)	Cell growth and proliferation	1.06	0.22	1.01E-06	0.00025
Protein of unknown function		1.06	0.22	2.29E-06	0.00045

Table 5: Supplementary information and References to Figure 4

Reference Nr in Figure 4	Species	Stressor	time	tissue	mRNA/ Protein	Reference	HSP70	
1	*Solea senegalensis*	pCO_2	1 month	pooled whole larvae	Protein	Pimentel et al., 2015	1.4849861	
1	*Solea senegalensis*	pCO_2	1 month	pooled whole larvae	Protein	Pimentel et al., 2015	2.1364074	*
2	*Carassius carassius*	hypoxia	7 day	brain	mRNA	Stenslokken et al., 2010	0.9610716	
2	*Carassius carassius*	hypoxia	7 day	heart	mRNA	Stenslokken et al., 2010	1.6777697	*
2	*Carassius carassius*	hypoxia	7 day	brain	mRNA	Stenslokken et al., 2010	11.9058795	*
3	*Paralichthys olivaceus*	salinity	2 weeks	liver	mRNA	Choi, 2010	29.3750244	*
3	*Paralichthys olivaceus*	salinity	2 weeks	liver	mRNA	Choi, 2010	53.3750634	*
3	*Paralichthys olivaceus*	salinity	2 weeks	liver	mRNA	Choi, 2010	55.8750634	*
4	*Mylio macrocepahlus*	salinity	8 month	liver	Protein	Deane et al., 2002	1.8479852	*
4	*Mylio macrocepahlus*	salinity	8 month	liver	Protein	Deane et al., 2002	0.4994684	
4	*Mylio macrocepahlus*	salinity	8 month	liver	Protein	Deane et al., 2002	1.6354201	*
5	*Sparus sarba*	salinity	1 month	liver	mRNA	Deane and Woo, 2004	0.9412418	
5	*Sparus sarba*	salinity	1 month	liver	mRNA	Deane and Woo, 2004	1.1841484	
5	*Sparus sarba*	salinity	1 month	liver	mRNA	Deane and Woo, 2004	1.0051117	
5	*Sparus sarba*	salinity	1 month	kidney	mRNA	Deane and Woo, 2004	1.0950025	
5	*Sparus sarba*	salinity	1 month	kidney	mRNA	Deane and Woo, 2004	0.9226692	
5	*Sparus sarba*	salinity	1 month	kidney	mRNA	Deane and Woo, 2004	0.8055263	
5	*Sparus sarba*	salinity	1 month	gill	mRNA	Deane and Woo, 2004	1.6199978	
5	*Sparus sarba*	salinity	1 month	gill	mRNA	Deane and Woo, 2004	0.3400002	*
5	*Sparus sarba*	salinity	1 month	gill	mRNA	Deane and Woo, 2004	0.3266668	*
7	*Paralichthys olivaceus*	salinity	48h	whole larvae	mRNA	Wu et al., 2017	1.3661964	
7	*Paralichthys olivaceus*	salinity	48h	whole larvae	mRNA	Wu et al., 2017	1.5563394	
3	*Paralichthys olivaceus*	temperature	2 weeks	liver	mRNA	Choi, 2010	49.6489394	*
3	*Paralichthys olivaceus*	temperature	2 weeks	liver	mRNA	Choi, 2010	46.3822024	*
1	*Solea senegalensis*	temperature	1 month	pooled whole	Protein	Pimentel et al., 2015	1.0679184	
1	*Solea senegalensis*	temperature	1 month	pooled whole	Protein	Pimentel et al., 2015	1.7344358	*
8	*Melanotaenia duboulayi*	temperature	14 days	liver	mRNA	Smith et al., 2013	9.9000000	*
This study	*Gadus morhua*	pCO_2	36 days	whole larvae	mRNA		1.6800000	*

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SUPPLEMENTARY INFORMATION CHAPTER 3

Table 1: pCO_2 treatments for individual life stages and corresponding in situ pH

Treatment	*Lifestage*	*Number of Samples*	Mean Temp. (°C)	*Standard Deviation*	*Mean A_T*	*Standard Deviation*	*Mean C_T*	*Standard Deviation*	*Mean pCO_2 (µatm)*	*Standard Deviation*	*Mean pH*	*Standard Deviation*
Ambient pCO_2	All stages	28	7.5	2.4	25.61	15.32	2139.12	10.22	439.50	43.52	8.01	0.04
	Parental incubation	5	4.6	0.3	37.20	13.31	2154.93	1.91	422.27	4.34	8.02	0.01
	Embryo	6	5.3	0.1	41.33	11.09	2139.99	2.27	384.73	17.55	8.06	0.02
	Larvae	17	9.1	1.7	16.65	9.65	2134.16	8.32	463.89	35.29	7.99	0.03
Increased pCO_2	All stages	29	7.6	2.4	30.59	17.27	2250.58	20.67	1027.41	150.18	7.67	0.06
	Parental incubation	5	4.6	0.4	46.00	9.92	2274.75	29.23	1091.51	282.80	7.64	0.10
	Embryo	6	5.3	0.1	45.17	6.82	2254.75	8.94	919.90	56.09	7.71	0.02
	Larvae	18	9.1	1.6	21.44	14.87	2242.48	15.23	1045.44	108.49	7.66	0.04

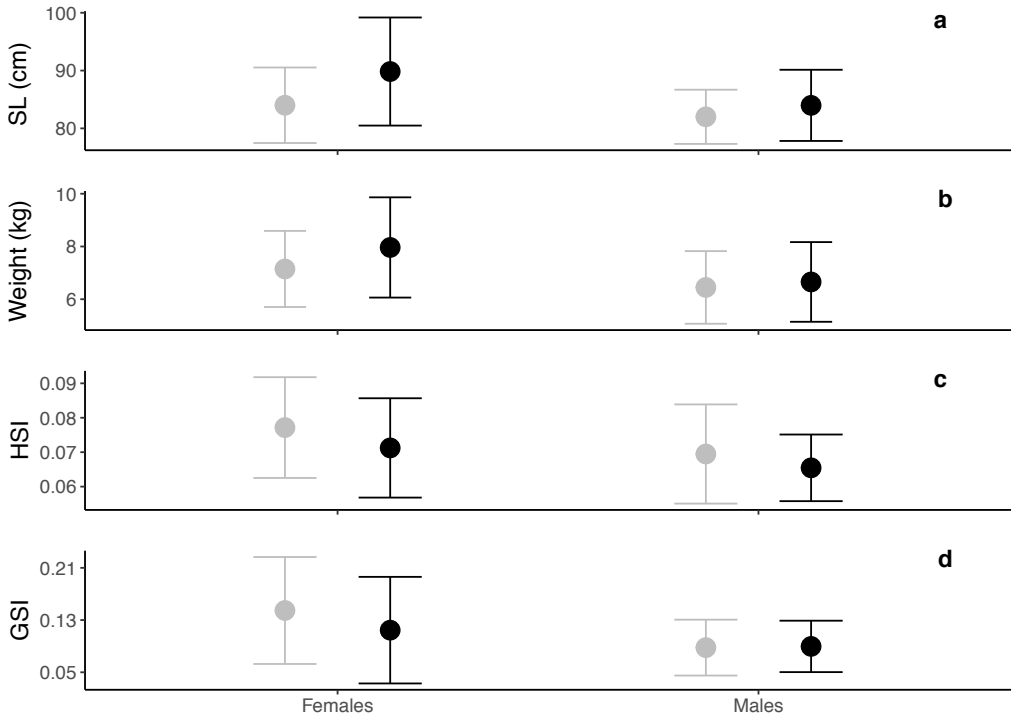

Figure 1: Parental fish in end April after 6 months of parental acclimation in ambient (grey) and increased (black) pCO_2, a) length, b) fresh weight, c) heptosomatic index (HSI) and d) gonadosomatic index (GSI). Centre is mean, whiskers SD.

Table 2: Parental fish after 5 months of incubation

Sex:	**Females**	N=34		**Males**	N=55	
Variable	**DF**	**t-ratio**	**p-value**	**DF**	**t-ratio**	**p-value**
Standard length (CM)	30,403	-2,1273	0,04162	45,544	-1,2639	0,2127
Weight (g)	31,286	-1,4174	0,1662	52,838	-0,05354	0,5947
GSI	31,507	1,072	0,2919	52,245	-0,16598	0,8668
HSI	31,421	1,1841	0,2453	45,286	1,2098	0,2362

Table 3: Parental condition (HIS) during incubation; 2-way ANOVA

Source of variation	**Degrees of freedom**	**F**	**P-value**
Treatment	1	1.515	0.220
Date	3	61.868	$<2x10^{-16}$
Treatment : Date	3	0.353	0.787

Table 4: arcsin Survival of larvae at 16 days post hatch; two-way ANOVA

Source of variation	**Degrees of freedom**	**F**	**P-value**
Parental treatment	1	31.722	$1.16*10^{5}$
Larval treatment	1	0.262	0.6135
Parental : Larval	1	3.928	0.0601

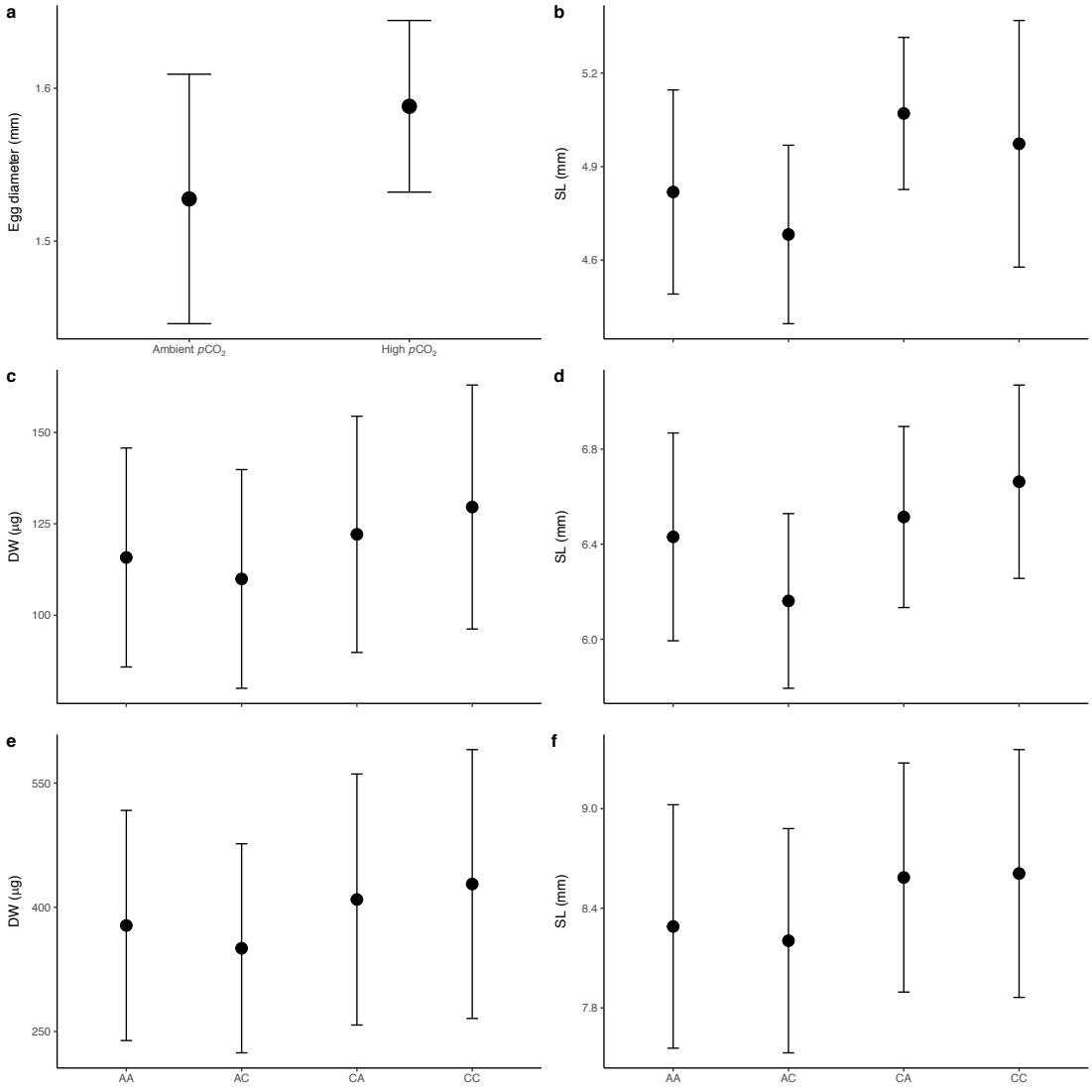

Figure 2: a) Egg diameter from ambient and high pCO_2 parents, b) Standard length (SL) in mm at 0 days-post-hatch, c) Dry weight (DW) in µg and d) Standard length (SL) in mm at 13 days-post-hatch, e) Dry weight (DW) in µg and f) Standard length (SL) in mm at 34 days-post-hatch. Centre are mean, whiskers are SD

Table 5: Statistics result for larval growth, dry weight (DW) and standard length (SL) using a linear mixed effects model with Parental and Larval treatment as fixed factor and larval tank as random factor

Age:	1 dph			13dph						34 dph					
Response:	SL^3			SL^2			DW				SL^3		√DW		
Source of variation	DF	F	p-value	DF	F	p-value	DF	F	p-value	DF	F	p-value	DF	F	p-value
Intercept	1	413.9053	$3.656x10^{-16}$	1	1924.7313	$< 2.2 x10^{-16}$	1	452.8184	$<2x10^{-16}$	1	503.8018	$<2x10^{-16}$	1	1107.8398	$<2x10^{-1}$
Parental Treatment	1	3.9098	0.06009	1	0.5792	0.453783	1	0.6024	0.4449	1	2.8866	0.1023	1	0.8817	0.3572
Larval Treatment	1	1.4341	0.24335	1	6.6863	0.016147	1	0.6320	0.4343	1	0.2666	0.6103	1	0.6754	0.4192
Parental: Larval Treatment	1	0.1064	0.74725	1	8.5804	0.007338	1	1.5749	0.2216	1	0.2477	0.6233	1	0.9676	0.3351

Table 6: Result summary for three-way ANOVA on Metabolic rate

Source of variation	Df	F	P-value
Parental treatment	1	10.327	0.00287
Larval treatment	1	0.894	0.35101
Age	1	3.679	0.06352
Parental treatment: larval treatment	1	1.062	0.31000
Parental treatment: age	1	3.716	0.06228
Larval treatment: age	1	0.073	0.78798
Parental treatment: larval treatment: age	1	0.435	0.51395

Table 7: Statistics result for larval condition, biochemical (RNA/DNA) and % soluble lipids (Lipids) using a linear mixed effects model with Parental and Larval treatment as fixed factor and larval tank as random factor

Response:	**RNA/DNA**			**Lipids**		
Source of Variation	**DF**	**F**	**p-value**	**DF**	**F**	**p-value**
Intercept	1	293.5477	1.768×10^{-15}	1	375.6253	$<2 \times 10^{-16}$
Parental treatment	1	0.0886	0.7683	1	1.3908	0.2492
Larval Treatment	1	0.4129	0.5269	1	0.0018	0.9664
Parental Treatment: Larval Treatment	1	0.0894	0.7676	1	0.6216	0.4382

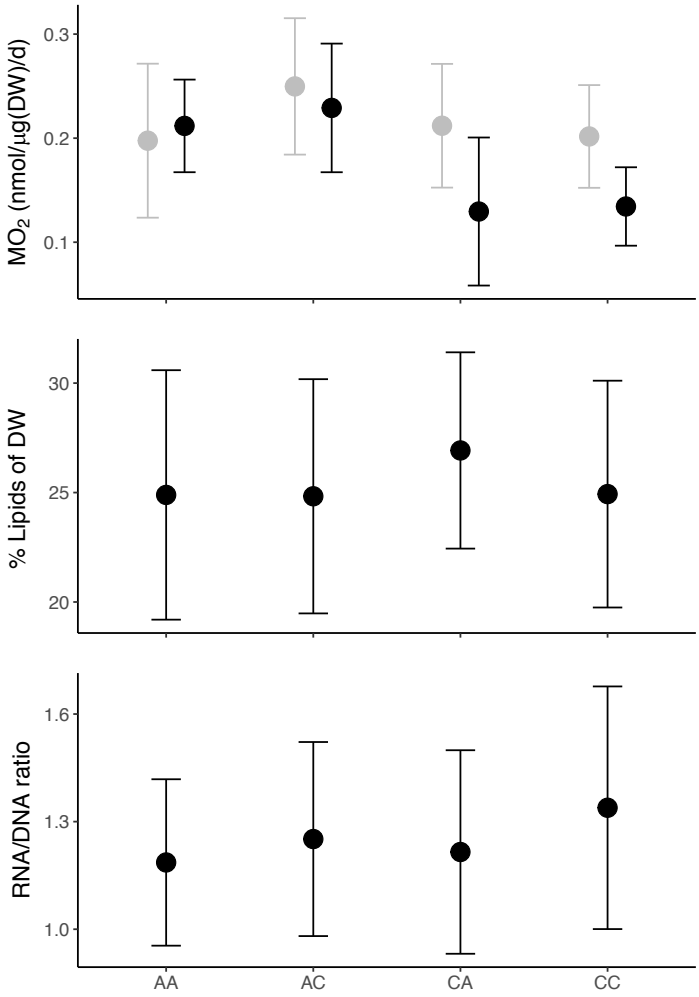

Figure 3: a) Metabolic rate of 10 (grey) and 11 (black) days-post-hatch larvae, b) % soluble lipids of dry weight (DW) of 34 days-post-hatch larvae, c) RNA/DNA ratio of 34 days-post-hatch larvae. Centre are mean, whiskers are SD

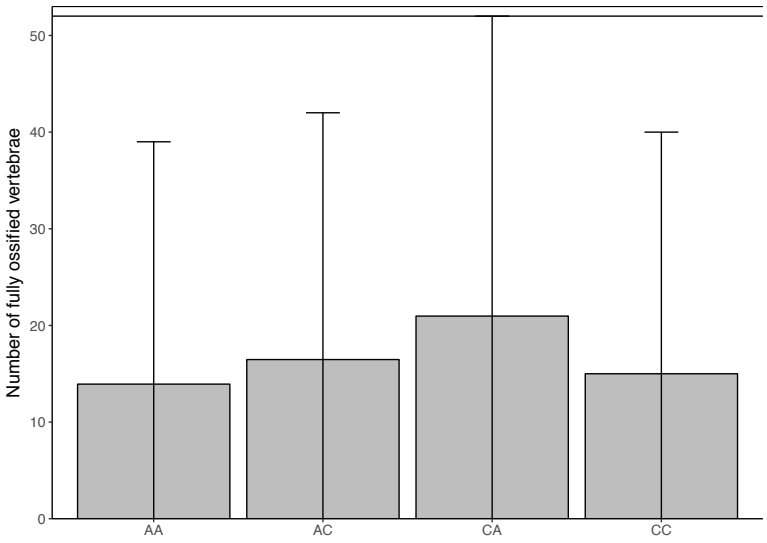

Figure 4: Number of fully ossified vertebrae. Bar is mean, whiskers are range.

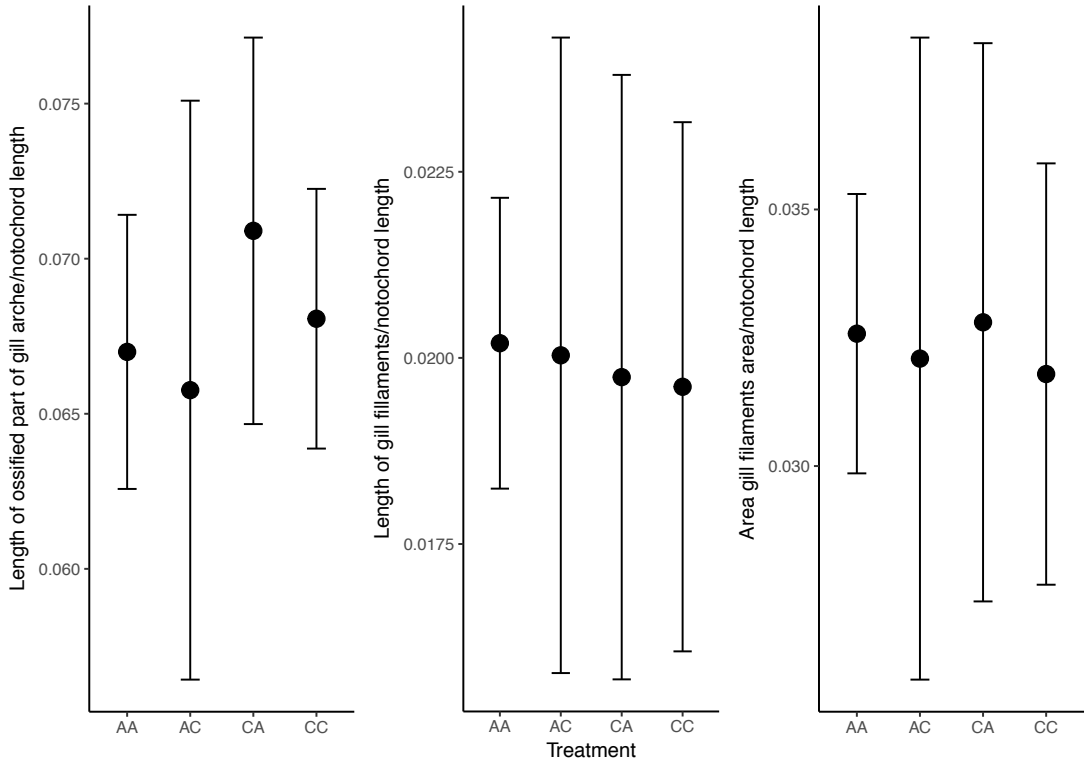

Figure 5: Length of a) ossified part of gill arch and b) longest gill filaments c) gill filament areas normalized to larval notochord length

Table 8: Statistics result for larval vertebrae ossification, using a general linear mixed effects model with Parental and Larval treatment as fixed factor and larval tank as random factor

Source of variation	Df	X^2	p-value(>X^2)
Intercept	1	18.8212	1.436×10^{-05}
Parental treatment	1	1.9477	0.1628
Larval treatment	1	0.3945	0.5299
Parental: Larval treatment	1	0.5891	0.4428

Table 9: Statistics result for larval gill development, Length of ossified gill arch, Length of longest gill filament and gill filament area, all normalized to notochord length using a linear mixed effects model with Parental and Larval treatment as fixed factor and larval tank as random factor

Response:	Ossified Arch Length			Filament Length			Filament Area		
Source of Variation	DF	F	p-value	DF	F	p-value	DF	F	p-value
Intercept	1	488.0284	$6.576x10^{-10}$	1	221.2683	$7.34x10^{-08}$	1	306.72	$1.597x10^{-08}$
Parental Treatment	1	0.7119	0.4183	1	0.1414	0.7154	1	0.0044	0.9485
Larval Treatment	1	0.2889	0.6015	1	0.1734	0.6852	1	0.1690	0.6890
Parental: Larval Treatment	1	0.6819	0.4264	1	0.0645	0.8043	1	0.2297	0.6412

Table 10: Summary of sequencing and mapping results

	raw reads			filtered reads		uniquely aligned reads		% aligned	
	sum	mean	SD	mean	SD	mean	SD	mean	SD
High pCO_2	$527.8x10^6$	$65.9 x10^6$	$3.2 x10^6$	$65,4 x10^6$	$3.1 x10^6$	$44,7 x10^6$	$2.2 x10^6$	68.33	1.70
Ambient pCO_2	$511.8 x10^6$	$63.9 x10^6$	$1.2 x10^6$	$63.5 x10^6$	$1.2 x10^6$	$42,7 x10^6$	$0.8 x10^6$	67.19	1.44
Total	$1.04 x10^9$	$64.9 x10^6$	$2.6 x10^6$	$64.4 x10^6$	$2,5 x10^6$	$43,7 x10^6$	$1.9 x10^6$	67.76	1.63

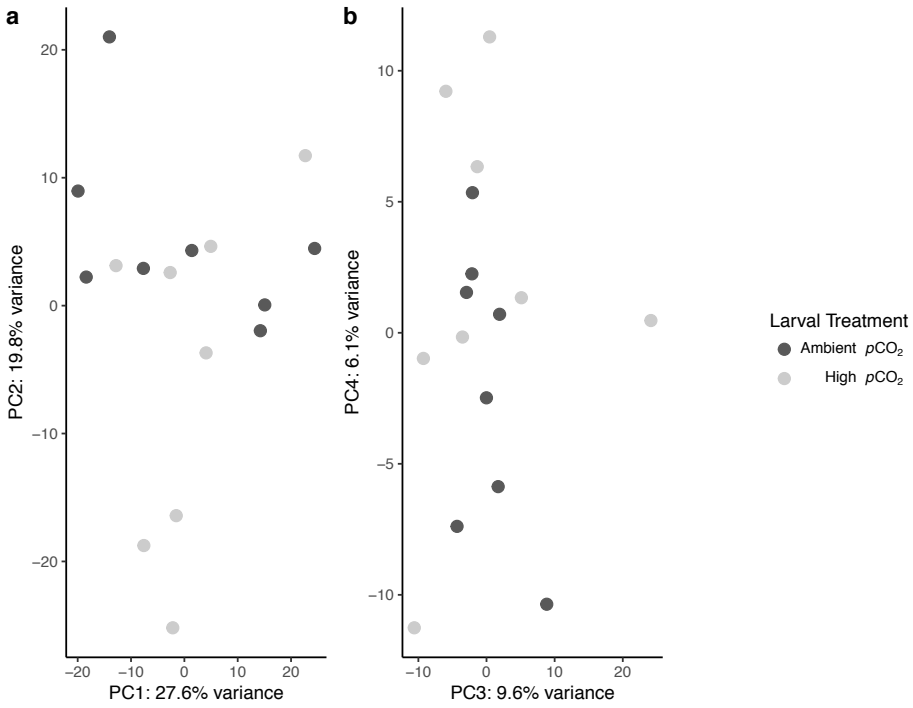

Figure 6: Unsupervised PCA based on quantification22790 annotations a) First and second principle component, b) third and fourth principle component

Table 11: Dunn's Kurskal-Wallis multiple Comparisons test on egg diameter

Comparison	Z	p-value	p-adjusted
Ambient pCO_2 –High pCO_2	-7.835789	$4.659076*10^{-15}$	$4.659076*10^{-15}$
Ambient pCO_2- 2014 Experiment	11.588879	$4.692330*10^{-31}$	$7.038496*10^{-31}$
High pCO_2- 2014 Experiment	17.496776	$1.516126*10^{-68}$	$4.548378*10^{-68}$

Table 12: Tukey's Post-hoc test on hepatosomatic Index (HSI) between acclimated fish from ambient and high pCO_2 and wild fish from mid-march 2014.

Comparison	Difference	Lower	Upper	p-adjust
Ambient pCO_2 –High pCO_2	-0.003533209	-0.01744428	0.01037786	0.8158356
Ambient pCO_2- 2014 Wild	-0.032229218	-0.04408763	-0.02037081	0.0000001
High pCO_2- 2014 Wild	-0.028696009	-0.03967794	-0.01771408	0.0000001

SUPPLEMANTARY INFORAMTION META-ANALYSIS

Survival data of Atlantic cod larvae after 16 days post hatch was extracted from Stiasny et al. (2016, submitted) and chapter 3. Means and SD for the larval control (ambient) and high pCO_2 was used to calculated the response (natural logarithm on response ration- ln RR). A meta-analysis was performed in OpenMEE (Wallace et al., 2017), applying a continuous random effects model using the DerSimonian-Laird method.

Table 1: Restults of Meta-analysis

Population	Parental Acclimation	Larval food Treatment	Study	Estimate	95% ci	Weight
Aquaculture	Ambient pCO_2	High	1	-1.549	(-2.418, -0.680)	8.163%
Aquaculture	Ambient pCO_2	Low	1	-0.512	(-1.229, 0.205)	9.992%
Aquaculture	High pCO_2	High	1	0.348	(-0.525, 1.222)	8.118%
Aquaculture	High pCO_2	Low	1	-0.720	(-1.092, -0.348)	15.339%
NEAC	none	High	2	-.733	(-1.422, -0.044)	10.367%
NEAC	none	Low	2	-1.061	(-1.842. -0.281)	9.182%
NEAC	Ambient pCO_2	Low	Chapter 3	0.049	(-0.159, 0.258)	17.734
NEAC	High pCO_2	Low	Chapter 3	-0.223	(-0.369, -0.078)	18.410%
Western Baltic	none	Natural	2	-2.727	(-4.579, -0.875)	2.695%
OVERALL I^2= 78.94%, P= 0.001				**-0.539**	**(-0.867, -0.211)**	

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