

**Influence of ocean acidification on otolith calcification
and behavior in fish larvae**

Dissertation

in fulfillment of the requirements for the degree "Dr. rer. nat."
of the Faculty of Mathematics and Natural Sciences
at Kiel University

submitted by
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Kiel 2012

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Date of the oral examination: October 24, 2012
Approved for publication: October 24, 2012

Signed: Prof. Dr. Wolfgang J. Duschl, Dean

Contents

| | |
|---|-----|
| Summary | ii |
| Zusammenfassung | iii |
| Introduction | |
| Temporal and spatial dimensions of ocean acidification | 1 |
| Influence of ocean acidification on the calcification of marine organisms.. | 3 |
| Otolith calcification and ocean acidification | 5 |
| Behavioral consequences of altered otolith calcification | 7 |
| Thesis outline | 9 |
| I Effects of ocean acidification on the calcification of otoliths of larval Atlantic cod, <i>Gadus morhua</i> L. (for resubmission in Marine Ecology Progress Series) | 13 |
| II The swimming kinematics of larval Atlantic cod, <i>Gadus morhua</i> L., are resilient to elevated seawater $p\text{CO}_2$ (published in Marine Biology Special Issue on Ocean Acidification) | 29 |
| III Effects of elevated $p\text{CO}_2$ on the swimming kinematics and foraging behavior of larval Atlantic herring, <i>Clupea harengus</i> L. (manuscript in preparation) | 51 |
| Additional preliminary data on Atlantic herring otoliths | 73 |
| IV Severe tissue damage in Atlantic cod larvae under increasing ocean acidification (second author, published in Frommel et al. 2012. Nature Climate Change 2:42-46) | 77 |
| Discussion | 91 |
| Outlook | 99 |
| Acknowledgements | 101 |
| References | 103 |
| Curriculum Vitae | 121 |
| Description of author contributions | 125 |
| Declaration | 127 |

Summary

The shift in the ocean's carbonate system towards a lower pH equilibrium, termed as ocean acidification, due to anthropogenic emissions of CO₂ has potential influence on the formation of calcium carbonate structures in marine organisms. This is because the precipitation of the calcified structures is dependent on seawater chemistry, with higher pH and aragonite saturation state favoring calcification. A number of studies reported different patterns of impact on calcification ranging from reduction to hypercalcification with some species showing no effects, which means that compensatory mechanisms are available to counteract the effects of reduced pH and elevated *p*CO₂. In marine fishes, the structures for mechanoreception known as otoliths are composed of aragonitic calcium carbonate and function for detection of motion and acceleration, maintaining balance, and sound localization. The morphology of the otoliths is species-specific and linked to the functional requirements for specific habitats and swimming behavior. In this thesis, the response of the otolith calcification in larval Atlantic cod (*Gadus morhua* L.) and Atlantic herring (*Clupea harengus* L.) to elevated *p*CO₂ and the possible consequences to the behavior of the larvae were investigated. The main results of the investigation were derived from an ocean acidification experiment conducted in the land-based mesocosm facility in the University of Bergen's Espegrend Marine Station from March to May 2010. Based on the results, the thesis concluded that otolith calcification in both Atlantic cod and herring larvae was significantly affected by increase in seawater *p*CO₂ concentrations. However, the direction of the effects was different between the two species with increase in otolith growth in cod larvae but a decrease in herring larvae. On the other hand, the changes observed in the otolith growth had no impact on the swimming behavior of both species. The swimming behavior was resilient to elevated *p*CO₂ despite the changes in the growth of the otoliths. Ocean acidification is not the only stressor associated with the increase in anthropogenic CO₂ emission. In marine fish larvae, the relationship between otolith morphology and swimming behavior must therefore be further investigated by considering additional stressors such as ocean warming, hypoxia, and fluctuations in food availability.

Zusammenfassung

Die Veränderung des Karbonat-Systems im Ozeans hin zu einem niedrigeren pH-Gleichgewicht, bekannt als Ozeanversauerung, und verursacht durch die anthropogenen CO₂-Emissionen, birgt ein großes Potenzial die Formation von Calciumkarbonat-Strukturen in Meeresorganismen zu beeinträchtigen. Dies begründet sich darin, dass die Ablagerung von kalzifizierten Strukturen von der Chemie des Meerwassers kontrolliert wird, wobei ein höherer pH-Wert und eine erhöhte Aragonit-Sättigung die Kalzifizierung begünstigen. Mehrere Studien beschreiben die verschiedenen Auswirkungen auf die Kalzifizierung, die von einer Reduktion bis zu einer Über-Kalzifizierung reichen. Bei einigen Meeresorganismen sind jedoch keine Auswirkungen zu erkennen, woraus sich schließen lässt, dass sie kompensatorische Mechanismen besitzen, die den Effekten eines erniedrigten pH-Wertes bzw. eines erhöhten $p\text{CO}_2$ entgegenwirken. Meeresfische besitzen Otolithen, Strukturen zur Mechanorezeption, die aus Aragonit, einem Calciumkarbonat bestehen, und die der Wahrnehmung von Bewegung, Beschleunigung, Geräuschen und dem Gleichgewicht dienen. Die Form der Otolithen ist artspezifisch und wird weitgehend geprägt von Lebensraum und Schwimmverhalten. In der vorliegenden Arbeit werden für larvalen Atlantischen Dorsch (*Gadus morhua* L.) und Atlantischen Hering (*Clupea harengus* L.) die Kalzifizierung der Otolithen und die möglichen daraus resultierenden Auswirkungen auf das Schwimmverhalten der Larven in Abhängigkeit von erhöhten $p\text{CO}_2$ untersucht. Die hauptsächlichen Ergebnisse der Untersuchungen stammen aus einem Experiment zur Ozeanversauerung, das in landgestützten Mesokosmen in der Meeresstation Espesrend der Universität Bergen, Norwegen, von März bis Mai 2010 durchgeführt wurde. Basierend auf diesen Ergebnissen wird abgeleitet, dass sich die Kalzifizierung der Otolithen sowohl bei atlantischen Dorsch- als auch bei Heringslarven mit steigenden $p\text{CO}_2$ -Konzentrationen im Meerwasser signifikant ändert. Jedoch sind die Effekte bei den zwei Arten gegensätzlich; beim Dorsch erfolgt eine Zunahme des Otolithen-Wachstums, beim Hering eine Abnahme. Eine auffällige Änderung im Schwimmverhalten konnte aber trotz der gravierenden Änderungen im Otolithen-Wachstum für beide Arten nicht festgestellt werden. Das larvale Schwimmverhalten beider Arten zeigte sich resilient gegenüber einem erhöhten $p\text{CO}_2$ trotz der Wachstumsänderungen in den Otolithen. Ozeanversauerung ist nicht der einzige Stressfaktor der mit den erhöhten anthropogenen CO₂-Emissionen in Verbindung gebracht werden sollte. Die Beziehung zwischen Otolith-Form und dem Schwimmverhalten sollte bei marinen Fischlarven daher detaillierter untersucht werden,

unter Hinzunahme weiterer Stressfaktoren wie Ozeanerwärmung, Sauerstoffarmut, und Fluktuationen in der Nahrungsverfügbarkeit.

Introduction

Temporal and spatial dimensions of ocean acidification

The continued increase of CO₂ concentration ([CO₂]) in the atmosphere by anthropogenic emissions from burning of fossil fuels, cement production, and other land-use practices such as deforestation has led to a phenomenon known as ocean acidification (Sabine et al. 2004; Olsen et al. 2006). Under this process, the carbonate system of the world's oceans has been modified resulting in a decrease in seawater pH and the subsequent alteration of the equilibrium of the rest of the carbonate system parameters: $p\text{CO}_2$, [CO₂], [HCO₃⁻], [CO₃²⁻], total CO₂ and total alkalinity (Zeebe and Wolf-Gladrow 2001; Ilyina et al. 2009; Zeebe 2012). This is because the oceans, which act as the only true net sink for anthropogenic CO₂ over the past 200 years, have absorbed about 48% of the anthropogenic CO₂ (Sabine et al. 2004). The dissolved inorganic carbon in seawater occurs in three forms, namely CO₂ (CO_{2aq} + H₂CO₃), HCO₃⁻, and CO₃²⁻ with [HCO₃⁻] about 6-10 times more than [CO₃²⁻] at normal pH above 8.0 (Kleypas et al. 1999; Pearson and Palmer 2000). The dissolved CO₂ forms carbonic acid, which readily dissociates into bicarbonate (HCO₃⁻), carbonate (CO₃²⁻) and proton (H⁺). The addition of anthropogenic CO₂ results in the increase production of H⁺ which consequently lowers the pH (Caldeira and Wickett 2003; Zeebe and Wolf-Gladrow 2001). A decrease of 0.1 pH unit or an equivalent of 30% increase in [H⁺] in oceanic seawater has been reported over the past 150 years (Caldeira and Wickett 2003). The additional H⁺ also binds to free carbonate ions (CO₃²⁻), causing a decline in the free carbonate ion concentration (IPCC 2001).

The current [CO₂] in the atmosphere of 380-390 parts per million by volume (ppmv) has increased by more than 80 ppmv relative to the maximum of 280 ppmv during the pre-industrial period (Petit et al. 1999; EPICA 2004; Hoegh-Guldberg et al. 2007). The increase in anthropogenic [CO₂] has been observed and quantified in several parts of the world's oceans. Increase in the partial pressure of CO₂ ($p\text{CO}_2$) in the Nordic seas from 1981-2003 has been attributed to the flow of Atlantic water from the south laden with high concentrations of anthropogenic $p\text{CO}_2$ and the inflow of low $p\text{CO}_2$ Polar Water, which enhances local uptake of atmospheric CO₂ in the Nordic Seas (Olsen et al. 2006). Increase of ocean anthropogenic CO₂ has already been observed in other regions as well, e.g. in the Indian Ocean from 1978-1995 (Peng et al. 1998), the Pacific Ocean from 1973-1996 (Peng et al. 2003), in the Southern Ocean's Antarctic Bottom Water from 1968-1996 (McNeil et al. 2001), and in bottom waters along the Indian-Atlantic

boundary (Lo Monaco et al. 2005). The projected increase (700-1000 ppmv) in atmospheric CO₂ concentration by the end of the century (IPCC 2001) is not unique and also not the highest relative to the geological past of the earth. Over the past 300 million years, atmospheric CO₂ levels have varied between 100-7500 ppmv (Caldeira and Wickett 2003). Atmospheric CO₂ concentrations relative to the present were 10-20 times higher between 570-350 million years ago (mya) in early Paleozoic and 2-6 times higher between 240-65 mya in the Mesozoic (Berner 1990). Lower than the present atmospheric CO₂ concentration are estimated for the late Paleozoic (330-260 mya) and over the last 30 million years in the late Cenozoic (Berner 1990). The carbonate system of the oceans has been relatively stable over the past 20 million years until the preindustrial period with *p*CO₂ maximum of about 280 ppmv and pH above 8 (Pearson and Palmer 2000).

Although the current projected increase in atmospheric CO₂ is not high relative to those estimated in the geological past, the rate of the increase in anthropogenic CO₂ is considered to be a cause for alarm. The rate of atmospheric CO₂ increase for the past 136 years (1870-2006) and for the year 2100 under the IPCC A2 business-as-usual scenario is 1050 and 6000 times faster than the rate over the past 420,000 years (Hoegh-Guldberg et al. 2007; Siegenthaler et al. 2005). Caldeira and Wickett (2003) have shown that ocean pH is more sensitive to additional CO₂ when that CO₂ change occurs over a short time interval of less than 10⁴ years than if it occurs longer than 10⁵ years. At these rates, the ocean's carbonate system coupled with slow oceanic mixing is seen to be less effective in buffering the reduction in pH thereby causing rapid changes that normally occur over thousands of years (Blackford and Gilbert 2007). In addition, the atmospheric CO₂ concentrations have been relatively stable between 180 and 280 ppmv over 400,000 to 650,000 years before the industrial period (Feely et al. 2004; Petit et al. 1999, Siegenthaler et al. 2005). Thus, the projected increase of up to 1000 ppmv by the year 2100 is quite unprecedented.

Many factors influence the solubility of CO₂ in seawater, and differences in physical oceanographic conditions and terrestrial freshwater inputs lead to high spatial and temporal variability in the sensitivity of the different oceanic basins and coastal zones to ocean acidification (IPCC 2001). For example, increased CO₂ solubility at low water temperatures, greater air-sea gas exchange as sea ice loss increases from global warming, CO₂-remineralization of high organic carbon load from seasonal primary production and low alkalinity riverine inputs have made the high latitude seas of the northern and southern hemispheres, and particularly the Arctic Ocean, potentially most

sensitive to ocean acidification (Fabry et al 2009; Bellerby et al. 2005). A decrease in pH by as much as 0.45 units (or an equivalent of 185% increase in $[H^+]$) over the 21st century is predicted for the Arctic surface waters (Steinacher et al. 2009). Upwelling of high pCO_2 seawater from intermediate and bottom depths expose marine communities along continental shelves and coastal zones to low pH waters much earlier than the climate change projections suggest for the open ocean. Low pH waters were measured from the surface until 450 meters deep in the eastern Bering Sea, and in the bottom waters inside Resurrection Bay and along the inner shelf of the Gulf of Alaska (Fabry et al. 2009). Upwelling of low pH subsurface waters along the northern coast of California has resulted in surface water pCO_2 of 850 μatm near the shelfbreak and higher pCO_2 inshore (Feely et al. 2008). In similar upwelling events in Kiel Fjord, surface pCO_2 values of greater than 2300 μatm were measured during summer and autumn (Thomsen et al. 2010; Melzner et al. 2012). Exposure of marine organisms to elevated pCO_2 as early as now due to upwelling events may elicit adaptive behavior and strategies to future acidification events. On the other hand, it may also start negative selection on organisms that cannot cope with stress from ocean acidification. Ocean acidification is a process that is changing conditions in the marine environment and we are just beginning to investigate how organisms adapt or not.

Influence of ocean acidification on the calcification of marine organisms

Acidification of the world's oceans has been identified as the other CO_2 problem in addition to global warming (Doney et al. 2009). High priorities have been allocated by researchers and policy-makers to investigate the impacts to calcifying marine organisms as the accompanying increase in $[H^+]$ lowers not only pH but also the available carbonate ions (CO_3^{2-}) (Fabry et al. 2008). Under normal seawater pH, carbonate ions precipitate readily with calcium (Ca^{2+}) ions into calcium carbonate ($CaCO_3$), which is used by many marine calcifying organisms to build external or internal structures (Guinotte and Fabry 2008). The reduction in the available carbonate ions in low pH condition makes the precipitation of $CaCO_3$ more difficult, thus affecting the calcification process.

The reason that decreasing available carbonate ions affects the calcification rate can be expressed by the saturation state. The calcium carbonate (aragonite or calcite) saturation state (Ω) is the ratio of the product of the in-situ concentrations of Ca^{2+} and CO_3^{2-} and the stoichiometric solubility product for $CaCO_3$ (Zeebe and Wolf-Gladrow 2001). Since $[Ca^{2+}]$ is fairly constant in seawater, the CO_3^{2-} is the molecule mainly

affecting the CaCO_3 saturation state (Kleypas et al. 1999). In an inorganic system, an Ω of 1 represents equilibrium between the dissolution and precipitation rates. Ω -values below 1 promote CaCO_3 dissolution while Ω -values above 1 favor precipitation. Most major marine calcifiers are found in waters with fairly high saturation state, e.g. nearly all shallow water coral reefs have $\Omega_{\text{aragonite}} > 3.25$ (Hoegh-Guldberg et al. 2007). Reduction of the optimal $\Omega_{\text{aragonite}}$ even though the values are still above 1 will push coral reef species from healthy to marginal conditions where formation of aragonitic exoskeleton becomes much more difficult (Guinotte et al. 2003). A similar situation could be faced by marine organisms located in areas sensitive to ocean acidification. It is projected that by 2070 with an atmospheric CO_2 concentration of 634 ppmv, the entire surface waters of the Arctic Ocean will be undersaturated with respect to aragonite ($\Omega_{\text{aragonite}} < 1.0$) and the whole water column by 2090 with atmospheric CO_2 of 765 ppmv (Steinacher et al. 2009).

A review of the literature on the effects of elevated $p\text{CO}_2$ in seawater on the calcification of wide range of marine calcifiers reveal variable responses (Doney et al. 2009). Ries et al. (2009) reported six general calcification response patterns to increase in $p\text{CO}_2$: 1) positive, 2) positive at highest $p\text{CO}_2$ only, 3) parabolic or positive at intermediate $p\text{CO}_2$ but negative at highest level, 4) neutral, 5) negative under highest $p\text{CO}_2$ only, and 6) negative. The wide variability in responses were attributed to several factors including regulation of pH at the site of calcification, protection of external shell by an organic layer, solubility of the CaCO_3 polymorph, and photosynthetic ability (Ries et al. 2009). Most of the studies with individual coral species (Gattuso et al. 1998; Marubini et al. 2001; Ries et al. 2009) and whole coral reef communities (Kleypas et al. 1999; Langdon et al. 2000, 2003; Leclercq et al. 2000) showed reduction in coral skeletal or net reef calcification. Reduced calcification was also reported in bivalves (Bechmann et al. 2011; Berge et al. 2006; Gazeau et al. 2007; Michaelidis et al. 2005; Ries et al. 2009), gastropods (Comeau et al. 2009, 2010; Ries et al. 2009), polychaete (Ries et al. 2009) and sea star and sea urchin (Gooding et al. 2009; Ries et al. 2009). On the other hand, enhanced calcification in response to elevated $p\text{CO}_2$ was reported in cephalopod (Gutowska et al. 2010b), cirriped barnacles (McDonald et al. 2009), malacostracans (Ries et al. 2009), and ophiuroid brittlestar (Wood et al. 2008). The increase in calcification in barnacles and brittlestars was identified as compensatory calcification to maintain skeletal integrity but at a cost resulting in weaker shells in barnacles and muscle wastage in brittlestar (McDonald et al. 2009; Wood et al. 2008). Reallocation of energy

for compensatory calcification may in fact lead to loss of the other functions of the parts being calcified. Although, high food consumption may counteract the reduction in calcification in high $p\text{CO}_2$ condition as well as maintain functional integrity of the calcified structures (Edmunds 2011; Melzner et al. 2011). The organism's ability to regulate the pH at the site of calcification by active acid/base regulatory mechanism could enhance calcification by some organisms when exposed to elevated $p\text{CO}_2$. When pH in seawater drops, extracellular pH drops also resulting in elevated HCO_3^- , which is the major form of dissolved inorganic carbon at low pH. If an organism is able to exclude the additional H^+ , the extracellular pH is maintained close to the normal pH allowing HCO_3^- ions to be converted into CO_3^{2-} promoting calcification (Ries et al. 2009). Organisms with active acid-base regulation such as cuttlefish and fish are able to elevate their blood and extracellular $[\text{HCO}_3^-]$ to maintain high pH (Gutowska et al. 2010a; Larsen et al. 1997).

Otolith calcification and ocean acidification

Marine fishes are not major CaCO_3 calcifiers. However, they produce otoliths that are made up of aragonite, a metastable CaCO_3 polymorph. Otoliths are equilibrium and hearing organs for fish (Tohse and Mugiya 2008). They are composed of 99% aragonite CaCO_3 precipitated continuously onto an organic matrix (Payan et al. 2004). The growth of the otolith is directly regulated by the carbonate composition and supersaturation states in the endolymph fluid, an isolated acellular environment in the closed sac of the inner ear (Tohse and Mugiya 2008).

The endolymph chemistry strictly controls the calcification process, which requires that all ionic and organic precursors for otolith formation are present in the endolymph (Payan et al. 2004). Specialized cells in the saccular epithelium synthesize the organic components while fluxes through the epithelium control the ionic composition. The ions in the endolymph are classified into two kinds namely, 1) the precursors of the CaCO_3 formation, which are either consumed (Ca^{2+} and HCO_3^-) or produced (H^+) during calcification and, 2) ions not directly involved in the calcification process (Na^+ , K^+ , Mg^{2+} , PO_4^{3-}) (Payan et al. 2004). The pH and bicarbonate in the endolymph are maintained at higher levels than those in the blood (Mugiya and Takahashi 1985; Payan et al. 1997, 1999; Tohse and Mugiya 2008).

Spatial variation in the concentration of ions has been reported between the proximal and distal spaces of the endolymph, which are separated by the otolith. The

proximal epithelium being freely permeable to ionic species involved in otolith growth causes higher concentrations of sodium, calcium, phosphate, magnesium and bicarbonate in the proximal endolymph while potassium, pH and total CO₂ are higher in the distal space (Payan et al. 2004). The ionic gradients are proposed to function as the driving forces that buffer the H⁺ produced during CaCO₃ formation and increase the supply of ionic precursors necessary to the major calcification region, which is the proximal side.

The endolymph contains excess amount of organic matrix precursors because only a small fraction (0.02-1%) of organic components are utilized in the daily otolith matrix formation (Payan et al. 2004). The organic compounds in the endolymph namely, 85% proteins, 12% collagens and 3% proteoglycans, are also not uniformly distributed with the proximal side having proteins, collagens and amino acids 4, 10, and 3 times more, respectively than those in the distal side. On the other hand, the proteoglycans are 10 times more at the distal side (Borelli et al. 2001) or undetectable in the proximal side (Payan et al. 2004). The same organic compounds and spatial variation along the proximo-distal axis are present in the otolith but in different proportions namely, 48% proteins, 23% collagens, and 29% proteoglycans. Borelli et al. (2003) proposed that collagen and protein gradients influence the organic matrix formation and otolith calcification process while Payan et al. (2004) admitted that the otolith matrix has significant role in the otolith crystallization processes of nucleation, orientation and growth control.

Based on the same aragonite saturation state equation, the mineral growth inside the endolymph relies on the concentrations of Ca²⁺ and CO₃²⁻, which in turn are influenced by several factors (Payan et al. 2004). Total [Ca], pH of the fluid, and nature and concentration of Ca-binding proteins affects [Ca²⁺] while [CO₃²⁻] depends on pH, partial pressure of CO₂, [HCO₃⁻], total [CO₂], dissolved [CO₂], and the solubility coefficient of CO₂. Calcium carbonate precipitates when the saturation ratio is greater than 1 and fish endolymph with $\Omega_{\text{aragonite}}$ of around 2-3 is considered a highly supersaturated fluid (Payan et al. 2004).

The amount of ambient CO₂ significantly affects the endolymph chemistry as Solomon et al. (2006) reported that the ratio of otolith carbon derived from metabolic-CO₂ and water is about 1:4. Tohse and Mugiya (2008) later reported that the rate of carbon incorporation from ambient water (0.79 nmol/mg otolith-h) is significantly higher than from metabolic-CO₂ (0.27 nmol/mg otolith-h) with the ratio of carbon contribution to the otolith from metabolic-CO₂ and ambient water being 1:3.

Thus, future changes in the carbonate system of the world's oceans are likely to influence the calcification of otoliths in marine fishes. The direction of the impact whether higher $p\text{CO}_2$ will result in reduced or enhanced calcification will depend on many factors but more importantly on the ability of the organism to regulate the endolymph pH, which also relies on the overall physiological condition of the larvae.

Behavioral consequences of altered otolith calcification

Elevated $p\text{CO}_2$ was previously shown by Checkley et al. (2009) to result in hypercalcification of otoliths in white seabass larvae but did not investigate its behavioral consequences. However, anecdotal report of lethargic swimming of white seabass larvae with hypercalcified otoliths was mentioned (Checkley and Dickson 2008 symposium abstract; Huelsenbeck 2010). Thus, the current thesis was undertaken to examine further the functional morphology of the otoliths in relation to potential changes in the behavior of fish larvae.

The otoliths are used by the fish as the primary organs for detection of body orientation, acceleration and sound localization or hearing (Tohse and Mugiya 2008; Lychakov and Rebane 2005). The main role of the otolith is to stimulate the sensory hair cells in the inner ear of the fish in response to body motion or external sound stimuli (Popper et al. 2005). Due to the density difference between the fish body and the otolith, the otolith establishes its own inertia relative to the body particularly when the fish accelerates or changes its swimming direction. The inertial difference is translated as a mechanical signal to the sensory hair cells. The mechanical signal created by the otoliths could be altered in case the optimum otolith dimensions or mass required for a certain larval length and swimming behavior is decoupled. For example, fast and agile swimmers usually have smaller otoliths relative to body size compared to sedentary and not so active swimmers (Popper et al. 2005). A fast and continuously swimming fish that constantly make changes in swimming direction may encounter problems with bigger otoliths because of the extra inertia that goes with the heavier mass. Similarly, a big otolith is conducive for less active swimmers because it needs the bigger mass to detect small changes in acceleration and change in position (Popper et al. 2005). Variations in the otolith calcification rate and general morphology resulting from elevated $p\text{CO}_2$ could potentially affect the behavior of fish such as in swimming, foraging for food and detection and escape from predators.

If elevated $p\text{CO}_2$ creates physiological stress to fish larvae, it could also increase the magnitude of fluctuating asymmetry (FA) between the paired otoliths, for example a bias of growth along one side of the fish (Somarakis et al. 1997). Fluctuating asymmetry, which is a measure of deviation from perfect bilateral symmetry, is a natural phenomenon in marine fish otoliths (Somarakis et al. 1997b; Lychakov and Rebane 2005). This means that the bigger-sized otoliths fluctuate between the right and left side throughout the life of a fish. However, it is known in marine teleosts that the magnitude of FA is maintained at low levels under normal growing conditions (Lychakov and Rebane 2005) but could increase due to stress (Somarakis et al. 1997). Fluctuating asymmetry was successfully used to compare the conditions of anchovy larvae sampled from different years (Somarakis et al. 1997b). Low levels of FA in the otoliths of anchovy larvae were attributed to superior larval conditions in 1994, in contrast to high FA and inferior larval conditions in 1995 (Somarakis et al. 1997b). Significant increase in the magnitude of FA has implications on the ability of the fish to react to sound stimulus since acoustic functionality such as sensitivity, temporal processing, and sound localization could be disturbed by increase in the asymmetry of the otoliths particularly in bigger otolith masses (Lychakov and Rebane 2005).

Thesis Outline

The thesis explored the influence of elevated $p\text{CO}_2$ and decrease in pH brought about by controlled $p\text{CO}_2$ perturbation experiments on the calcification of otoliths and possible behavioral consequences in marine fish larvae. Its major aim is to examine whether changes in the calcification of the otoliths arise from increase in seawater $p\text{CO}_2$, and whether such changes will be reflected in the swimming behavior. The thesis comprises of four chapters, each briefly described below. The results presented here were derived from a land-based mesocosm ocean acidification experiment conducted from March to May 2010 at the Espegrend Marine Station of the University of Bergen in Norway. The larvae used in the experiment were grown under three $p\text{CO}_2$ concentrations: 370, 1800, and 4200 μatm .

Chapter I

Effects of ocean acidification on the calcification of otoliths of larval Atlantic cod, *Gadus morhua* L.

(In revision for Marine Ecology Progress Series)

This chapter focuses on how the structure and form of otoliths of Atlantic cod larvae from 7 to 46 days post hatch (dph) respond to different $p\text{CO}_2$ conditions. Both pairs of the sagitta and lapillus otoliths were analyzed. The proxy for otolith growth and calcification was obtained from the analysis of the otolith area normalized to fish standard length. It was evident from the results that elevated $p\text{CO}_2$ resulted in otoliths with much wider otolith area. The chapter also reports no change on the magnitude of fluctuating asymmetry between the paired otoliths.

Chapter II

The swimming kinematics of larval Atlantic cod, *Gadus morhua* L., are resilient to elevated seawater $p\text{CO}_2$

(published in 2012 Marine Biology Special Issue on Ocean Acidification)

Building up on the clear significant increase in otolith calcification due to elevated $p\text{CO}_2$ as reported in Chapter I, the thesis proceeds to investigate the functional morphology of the otoliths, i.e. the behavioral consequences for the Atlantic cod larvae of having bigger otoliths. A 3-dimensional silhouette video photography (SVP) was used to examine the fine details of the swimming kinematics of the larvae, which include swim duration, distance and speed, stop duration, and turn angles along the horizontal

and vertical axes relative to the body of the fish. Atlantic cod larvae aged 12 and 27-dph were used for the analysis of the swimming behavior. The chapter reports that the swimming kinematics of Atlantic cod larvae were quite resilient against increase in $p\text{CO}_2$ concentrations despite the significant increase in otolith growth.

Chapter III

Effects of elevated $p\text{CO}_2$ on the swimming kinematics and foraging behavior of larval Atlantic herring, *Clupea harengus* L.

(manuscript in preparation)

This chapter examines how the larval swimming behavior of another marine fish species, Atlantic herring, respond to the same increase in $p\text{CO}_2$. The same 3d-SVP procedure was used to examine the swimming kinematics of 34 and 40-dph larvae. The chapter also reports that herring larval swimming behavior at the ages observed were also robust to increase in $p\text{CO}_2$ despite the fact that elevated $p\text{CO}_2$ resulted in significantly smaller larvae with slight delay in ontogenetic development. The swimming behavior of Atlantic herring larvae reported here could only represent the individuals that survive a possible high mortality event that occurred at 25-dph, as inferred from severe tissue damages observed. This however, provided an opportunity to examine the functional morphology of the otoliths in the absence of histological damages. In contrast to increase in otolith growth observed in Atlantic cod reported in Chapter I, Atlantic herring larvae had significantly smaller otoliths at elevated $p\text{CO}_2$ conditions (data provided in the Additional Preliminary Results section). Due to the conspicuous feeding strikes of herring larvae, observations on the foraging behavior of herring larvae were included.

Chapter IV

Severe tissue damage in Atlantic cod larvae under increasing ocean acidification

(second author, published in Frommel et al. 2012. Nature Climate Change 2:42-46)

This chapter reports on the biochemical and histological examinations conducted on the Atlantic cod larvae. Severe tissue damages in the liver, kidney, pancreas, eye and gut, increase in lipid content and dry weight of larvae were observed in the elevated $p\text{CO}_2$ treatments at 32-dph. At 32-dph, cod larvae underwent major restructuring and development of organs, which is composed of energetically costly processes that were likely disrupted by increase in $p\text{CO}_2$. Severe tissue damages were absent in larvae

younger than 32-dph. Dry weight, standard length, and fish condition based from RNA/DNA were also not significantly different among the $p\text{CO}_2$ treatments in the larvae younger than 32-dph. This chapter highlights the fact that Atlantic cod larvae were vulnerable to elevated $p\text{CO}_2$ at specific developmental stages particularly at 32-dph. However, the non-significant effects in the younger larvae provided the opportunity to examine the functional morphology of the otoliths in the absence of tissue damage that would otherwise complicate the behavioral analysis. The only major significant response of Atlantic cod larvae at ages younger than 32-dph was the increase in otolith growth as discussed in Chapter I.

Chapter I

Chapter I

Effects of ocean acidification on the calcification of otoliths of larval Atlantic cod,
Gadus morhua L.

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Abstract

The growth and development of the aragonitic CaCO₃ otoliths of teleost fish could be vulnerable to processes resulting from ocean acidification. The potential effects of an increase in atmospheric CO₂ on the calcification of the otoliths were investigated by rearing Atlantic cod larvae (*Gadus morhua* L.) in three pCO₂ concentrations, control-370, medium-1800, and high-4200 µatm from March to May 2010. Increased otolith growth was observed in 7 to 46-day post hatch (dph) cod larvae at elevated pCO₂ concentrations. The sagittae and lapilli were usually largest at the high pCO₂ treatment followed by medium and control treatments. The biggest difference in mean otolith surface area (normalized with fish length) was for sagittae at 11-dph with medium and high treatments being 46% and 43%, respectively, larger than the control group. On the other hand, Atlantic cod larvae showed no trends in the fluctuating asymmetry of the otoliths, defined as the difference between the right and left sides, in relation to the increase in otolith growth from elevated pCO₂. There was also no clear pCO₂ effect on the shape of the otoliths.

Keywords: otolith growth/calcification, ocean acidification, Atlantic cod larvae, *Gadus morhua* L.

Introduction

The Atlantic cod (*Gadus morhua* L.) is a northern temperate species, living in a region that is projected to be highly sensitive to future increases in atmospheric carbon dioxide. This is due to higher solubility of CO₂ at low water temperatures, increased air-sea exchange as sea ice loss increases from global warming, CO₂-remineralization of high organic carbon load from seasonal primary production and low alkalinity riverine inputs (Fabry et al. 2009). Upwelling events in coastal waters could worsen the levels of ocean acidification as it brings to the surface corrosive acidic waters with remineralized CO₂ as reported in the bay and inner shelf of the Gulf of Alaska in September 2008 (Fabry et al. 2009) and in Kiel Fjord during summer and autumn in 2008 (Thomsen et al. 2010). The major spawning area of the Arcto-Norwegian cod stock of *Gadus morhua* in the Vestfjorden in the Lofoten archipelago in Norway is also subject to wind driven upwelling and downwelling events (Furnes and Sundby 1981). Upwelling caused by winds blowing from the southwest spread the eggs out towards the central part of Vestfjorden while downwelling due to winds from the northeast concentrates the older egg stages towards the shore. Wind-driven upwelling and downwelling events along the Norwegian coast also play a role in the dispersal and retention of planktonic organisms and early life stages of fish between the fjords and the Norwegian Coastal Current (Asplin et al. 1999). Accumulation of high *p*CO₂ bottom water, which can be upwelled to the surface, along the Norwegian Sea and Barents Sea is also possible. Both regions act as carbon sinks from the first part of the year until the end of summer (Findlay et al. 2008; Bates et al. 2009).

Although teleost fishes are not major CaCO₃ calcifiers, they produce otolith structures that are mainly composed of aragonitic CaCO₃ (Degens et al. 1969). This potentially makes the otolith structure sensitive to elevated levels of *p*CO₂. Munday et al. (2011a) discussed three possible consequences of ocean acidification on otolith calcification. First, fish might have more difficulty in precipitating aragonite as available carbonate ion concentration declines in seawater. Second, otolith hyper-calcification might result from increased concentrations of carbonate and bicarbonate ions inside the endolymph due to the maintenance of internal pH by the fish regulatory mechanism with increased ambient *p*CO₂. Third, physiological stress from acidic conditions could indirectly influence otolith shape and symmetry. Otolith hyper-calcification was reported in white sea bass larvae (*Atractoscion nobilis*) at 993 and 2558 ppm of CO₂ (Checkley et al. 2009), and clownfish larvae (*Amphiprion percula*) at 1721 ppm (Munday et al.

2011a). On the other hand, otolith calcification was not affected in spiny damselfish juveniles (*Acanthochromis polyacanthus*) grown at 850 ppm of CO₂ (Munday et al. 2011b).

In this study, the influence of elevated seawater $p\text{CO}_2$ and the associated shift in carbonate chemistry equilibrium on the calcification of the otoliths of early life stages of Atlantic cod was investigated. The morphology of the otoliths is highly important for the normal acoustic and behavior functions in fish (Gauldie 1988, Aguirre 2003, Lombarte 1992), and abnormal otoliths may ultimately represent an added mortality risk. Understanding the influence of external environmental factors on the growth of the otoliths is also important because otoliths are widely used in fish studies such as age determination, larval fish ecology and growth studies (Campana 2005).

Materials and methods

Seawater manipulation

The experiment was conducted in the land-based mesocosms at the University of Bergen's Espesrend Marine Station from March to May 2010. Computer-controlled bubbling of CO₂ into the bottom of nine 2650-L experimental tanks was done to achieve three $p\text{CO}_2$ levels: control (370 ppm), medium (1800 ppm) and high (4200 ppm), with three replicates for each treatment level (see supplementary Table 4 in Frommel et al. 2012). A stable rise of temperature from 5°C in March to 10°C in May was achieved by placing the 2650-L tanks inside two water baths, which minimized the influence of fluctuations in air temperature. The gradual rise in temperature with mean salinity of 33.3 psu reflected the natural increase in water temperature of the fjord at 40-m depth which was the source of the water supplied to the experimental tanks using a flow through system. For further details, see Frommel et al. (2012).

Larval rearing

Newly fertilized Norwegian coastal cod eggs sourced from Parisvatnet Field Station of the Institute of Marine Research were transferred to the floating incubation buckets in the experimental tanks on March 25, 2010. The eggs remained under normal $p\text{CO}_2$ conditions for three days, after which the $p\text{CO}_2$ levels were adjusted to the targeted $p\text{CO}_2$ for each treatment. Fifty percent hatching occurred on April 9, 2010 and was designated as 0-day post hatch (dph). Redistribution of hatched larvae among replicates within each treatment was carried out on 0-dph to give an equal stocking density of 4 larvae L⁻¹ in each tank.

Feeding density of 2000 prey L⁻¹ of natural zooplankton was maintained by 24-h filtration of adjacent seawater using the Hydrotech size-selective filter system (Seljeset et al. 2010). Initially, the larvae were fed with zooplankton size fraction of 80-250 µm and gradually increased to 350-500 µm.

Handling of animals in the mesocosm-experiment was conducted using the animal experimentation permit ID2346 granted by the Animal Welfare Committee as determined under the Norwegian Animal Welfare Act.

Fish sampling and otolith preparation

Fish larvae were sampled eight times from 7-dph until 46-dph. Sampling through the whole water column was done by dropping a PVC-pipe with a manual closing mechanism to the bottom of the tanks obtaining 3-12 larvae in each scoop. Point sampling close to the water surface was later implemented when it became harder to catch the larvae using the PVC-pipe. Each fish larva was photographed in a thin film of seawater in less than 30 seconds and frozen individually in Eppendorf tubes at -80°C. Standard lengths of the fish larvae were later measured using the calibrated pictures. The lapilli and sagittae were dissected from the fish larvae using fine needles and mounted on glass slides using Crystalbond™ 509. Pictures were taken of each otolith using an Olympus BX61 compound microscope. Most of the pictures of the otoliths were taken using an oil immersion objective (total magnification 1000x) while larger otoliths from older larvae were taken at 200-600x total magnification. The otolith surface area and dimensions were measured from the pictures of the otoliths using the ImageJ software (Wayne Rasband, National Institutes of Health USA).

Analysis

The otolith surface area was compared among treatments by Analysis of Covariance (ANCOVA) with fish standard length used as the covariate to account for differences in fish length among fish larvae and dependence of otolith size on fish size (Otterlei et al. 2002). Data from all the larvae in the three replicate tanks of each treatment were treated as one group because there were no significant differences among the replicates within each treatment. Otolith surface area was log-transformed in some age groups (32 and 46-dph) in order to meet the ANCOVA assumption for homogeneity of regression slopes among the three pCO₂ treatments, which is required to test for intercept (otolith area) differences. As for the rest of the age groups, the regression slopes among treatments were not significantly different.

In Atlantic cod, an ontogenetic shift in the growth rates of the sagitta and lapillus takes place with bigger lapilli at the earlier larval stages and then a shift to bigger sagittae. For example, Bergstad (1984) reported the shift in the growth rates of sagitta and lapillus at about 25-dph or 6-mm standard length. This transition was monitored in this study by taking the ratio of the surface area of the sagitta versus lapillus for each fish larva. Comparison of the sagitta/lapillus surface area ratio among $p\text{CO}_2$ treatments per sampling date was done using Nested-design Analysis of Variance (Nested-ANOVA), using tanks nested in $p\text{CO}_2$ treatments. A non-parametric Kruskal-Wallis test was performed for sampling dates with sagitta/lapillus surface area ratio that did not satisfy assumptions for normality and homogeneity of variance. The same procedure for Nested-ANOVA and Kruskal-Wallis test was performed on fish standard length.

Differences in otolith surface area between the right and left sides referred to as fluctuating asymmetry were derived for each larva when both right and left otoliths were available (Somarakis et al. 1997). Higher variability was hypothesized in the high CO_2 treatment. The differences between the two sides were normalized by dividing with the mean otolith surface area of the two sides and then plotted against age of the larvae. The homogeneity of variances of the fluctuating asymmetry with age among the $p\text{CO}_2$ treatments was tested using Hartley F-max tests of homogeneity of variances.

The shape of the lapillus and sagitta was also compared using the roundness parameter derived from the otolith pictures using the ImageJ software. A value of 1 means a perfect circle while values approaching 0 indicate increasingly elongated shape (Geffen, FishPopTrace report). A Nested-Design Analysis of Variance was used to compare otolith roundness among the $p\text{CO}_2$ treatments with replicate tanks nested in each treatment. Individual roundness value from both the left and right side otoliths was included in the analysis.

Results

Increase in growth of both lapillus and sagitta otoliths were observed from 7 until 46-dph cod larvae (Figure 1). Larger otolith surface areas were observed from Atlantic cod larvae in the high treatment followed by medium and then control treatments. The otolith surface area of the medium treatment fluctuates between control and high treatments. The biggest difference in mean otolith surface area (ANCOVA-adjusted with fish length) was at 11-dph when the sagittae of larvae from the medium and high treatments were 46% and 43%, respectively, larger than the control group.

An ontogenetic shift in the growth rates of the lapillus and sagitta was observed in all larvae (Figure 2). The lapillus was initially larger than the sagitta, then the growth of the sagitta overtook that of the lapillus. However, the timing of the shift in the sagitta/lapillus surface area ratio occurred earlier, at 32-dph, for larvae from the high treatment than larvae from control and medium treatments. At 11 and 18-dph larvae, the high treatment already showed significantly higher sagitta/lapillus surface area ratio than control and medium treatments even though the shift in growth rates had not yet occurred. In relation to the mean standard length, the larvae from the high treatment already surpassed the 6 mm standard length and was significantly longer than the control and medium treatments at 32-dph (Figure 3). There were also some signs of an increase in standard length with increase in $p\text{CO}_2$ as shown at 11, 25, and 32-dph.

The side with larger-sized otoliths fluctuated between the right and left side (Figure 4), but there was no bias in the growth rates of either side giving no indication of directional asymmetry or increase in the magnitude of the fluctuation. Variances of the differences in otolith area between right and left sides with age were homogenous amongst $p\text{CO}_2$ treatments (Hartley F-max: lapillus 1.28, sagitta 2.33, $p > 0.05$).

The mean roundness values of the lapillus from 7-dph until 39-dph range from 0.91 to 0.94 without significant differences among the $p\text{CO}_2$ treatments (Nested-ANOVA, $p > 0.05$) (Figure 5). By 46-dph, the mean roundness values of the lapillus decreased significantly compared to the younger larvae with otoliths from the medium and high treatments having significantly more elongated shape than those from the control group (Nested-ANOVA, $p < 0.001$). For the sagittal otoliths, the roundness values increased from 7-dph until 32-dph but became more elongated again at 46-dph. There was no clear $p\text{CO}_2$ treatment effect on the shape of the sagitta except at 25-dph where more rounded sagittae were associated with increased $p\text{CO}_2$ (Nested-ANOVA, $p = 0.016$).

Discussion

In this study, we have shown that otoliths of Atlantic cod larvae were larger at elevated $p\text{CO}_2$ conditions. Increase in otolith growth began early in development and was observed even in the youngest sampled larvae at 7-dph up until 46-dph. Several studies have already explored the possibility of enhanced calcification under elevated $p\text{CO}_2$ conditions in marine organisms with active acid/base regulatory mechanism. High ambient $p\text{CO}_2$ could result to respiratory acidosis where extracellular $p\text{CO}_2$ rises and pH decreases (Melzner et al. 2009b). In organisms with high acid-base regulation,

respiratory acidosis is counteracted by elevating the levels of bicarbonate (HCO_3^-) in the blood and extracellular spaces, which in turn brings the pH closer to the normal level (Melzner et al. 2009b; Marshall and Grosell 2005). Cuttlefish minimized the decrease in extracellular blood pH by only 0.18 units when subjected to acute hypercapnia at 0.60 kPa $p\text{CO}_2$ (Gutowska et al. 2010a). This extracellular pH regulation in cuttlefish was achieved through active ion-transport processes, which rapidly increased extracellular HCO_3^- from 3.38 mM to 9.8 mM within 8h and stabilized at 10.4 mM after 24h. Similarly, Atlantic cod (230-525 g) showed an increase of 21 mM HCO_3^- in the blood over 24h when exposed to 1.10 kPa (Larsen et al. 1997). The increase in HCO_3^- in the blood of cuttlefish and the partial compensation of extracellular pH could have increased the CaCO_3 saturation state, thereby resulting in elevated calcification rates of the cuttlebone under long-term elevated $p\text{CO}_2$ conditions (Gutowska et al. 2008, Gutowska et al. 2010b). In teleost fishes, Payan et al. (1998) demonstrated that blood pH in unstarved fish shows a significant positive relationship with the endolymph pH, while Mugiya and Takahashi (1985) reported higher pH and total CO_2 in the endolymph compared to plasma. Thus, maintenance of high pH in the blood during hypercapnia could also be translated into higher pH in the endolymph. The hypercalcification observed in larval clownfish, *Amphiprion percula*, at 1721 $\mu\text{atm } p\text{CO}_2$ (Munday et al. 2011a) and in white seabass larvae at 993 and 2558 $\mu\text{atm } p\text{CO}_2$ (Checkley et al. 2009) were attributed to pH regulation in the otolith endolymph. Payan et al. (1998) provided the evidence that at endolymph pH of 7.7 to 8, most of the total CO_2 is in HCO_3^- form, which acts as the main source for CaCO_3 precipitation onto the otoliths. Thus, this could be the mechanism causing the increase in otolith growth at elevated $p\text{CO}_2$ levels in our study.

Acid-base regulation by branchial or gill respiration was not yet functional in most of the cod larval stages in our study especially in the younger larvae. Structures that aid in effective gill ventilation such as the opercular bones start to form only at 40-50-dph (Hunt von Herbing et al. 1996). However, the increase in otolith growth in cod larvae at elevated $p\text{CO}_2$ could point to an already effective acid-base regulation by cutaneous respiration in the early larval stages. Acid-base regulation by raising the HCO_3^- concentrations might be just enough for the pH compensation required to counteract acidosis and consequently caused more precipitation of calcium carbonate into the otolith structure. Functional acid-base regulation even at early stages in cod larvae could have been aided by the presence of accessory respiratory structures such as

the pseudobranch, a structure with large volume of blood cells and thin epithelial lining positioned in the head of cod larvae, and pigmented haemoglobin in the red blood cells (Hunt von Herbing et al. 1996; Matthey et al. 1978; Marshall and Grosell 2005).

Otoliths are the main organs for detecting acceleration, balance and sound in fish. Increase in the growth of otoliths due to elevated $p\text{CO}_2$ could have consequences on the otolith functions. Huelsenbeck (2010) reported less swimming activity in white seabass larvae grown at 2500 ppm of CO_2 coincident with occurrence of hyper-calcified otoliths. However, cod larvae aged 12-dph and 27-dph from our experiment showed only subtle changes in their swimming behavior due to increased $p\text{CO}_2$ concentration (Maneja et al. 2012). Changes in otolith growth due to projected and extreme local ocean acidification events might not translate to behavioral changes in fish larvae. Reports on larvae of tropical marine fishes point to disruptions of neurotransmitter functions in the brain due to elevated levels of bicarbonate (HCO_3^-) concentrations in the blood as the main cause of behavioral changes under ocean acidification events (Nilsson et al. 2012). The behavioral changes in fish larvae included damage to olfaction causing loss of homing ability (Munday et al. 2009b), loss of detection and avoidance against predator (Munday et al. 2010, Dixson et al. 2010), and loss of predatory reaction to presence of prey (Cripps et al. 2011). The impairment of the neurotransmitter function might have also affected the ability of the larvae to decide between left and right turns (Domenici et al. 2012) and the loss of ability to learn anti-predatory responses (Ferrari et al. 2012).

Another interesting result from the study was the earlier transition of the growth rates of the sagitta and lapillus in the high treatment, which occurred at 32-dph, compared to the medium and control treatments at 39-dph. The differences in the timing of transition in the growth rates of the lapillus and sagitta could have been an indirect effect of the observed differences in the somatic growth rate of the larvae. Bergstad (1984) reported that lapilli of cod larvae reared at $5\pm 1^\circ\text{C}$ were always larger than the sagittae from hatching until a standard length of approximately 6 mm or at around 25-dph. Dale (1984) also reported bigger lapillus than sagitta in early larval stage of cod. In the current study, the mean standard length of the larvae from the high treatment already surpassed the 6 mm standard length and was significantly longer than the control and medium treatments at 32-dph. Cod larvae from the high treatment also had significantly heavier dry weights than fish in the control group at 32-dph (Frommel et al. 2012).

Although increased otolith growth was observed in both lapillus and sagitta at elevated $p\text{CO}_2$ levels, there was no systematic pattern of deviation from the normal

fluctuating asymmetry and no severe changes in otolith shape. The cod larvae were able to keep a similar and low magnitude of fluctuation of otolith growth between the right and left sides (fluctuating asymmetry) in all treatments. These findings support the earlier report by Munday et al. (2011a) on the maintenance of otolith symmetry and shape despite increase in otolith growth at 1721 $\mu\text{atm } p\text{CO}_2$. This could also mean that changes in the carbonate chemistry inside the endolymphs occur at the same magnitude in both left and right sides. The subtle deviations from bilateral symmetry in otolith size has been recommended as an indicator of condition in larval fish, where negative reactions to stress events are recorded as increased levels of fluctuating asymmetry (Somarakis et al. 1997). Lychakov and Rebane (2005) suggested that a specific physicochemical mechanism of the paired otolith growth must exist, which allows teleost fishes to minimize the fluctuation in otolith masses between the left and right otoliths for proper acoustic functionality. Acoustic functionality of the fish such as sensitivity, temporal processing, and sound localization were shown through mathematical modelling to be affected by increasing otolith mass asymmetry particularly those involving large otolith masses.

In summary, the current study showed that conditions mimicking global and locally-enhanced ocean acidification influenced the growth characteristics of the otoliths of the early larval stages of Atlantic cod, causing increased otolith growth and size-related earlier transition in lapillus and sagittal growth rates. However, the current study did not show increase in the magnitude of fluctuating asymmetry or lead to directional bias in otolith growth as well as severely damage the shape of the otoliths. The increase in otolith calcification due to elevated $p\text{CO}_2$ might be well within the natural variation in otolith sizes that will cause no impairment in the functions of the otoliths.

Acknowledgements

Funding support was provided through the European Marie Curie Initial Training Network "Calcification by Marine Organisms" (CalMarO) and the European Community's Seventh Framework Programme (FP7/2007-2013) "European Project on Ocean Acidification" (EPOCA, grant agreement N211384). The study was also supported by the project "Biological Impacts of Ocean ACIDification" (BIOACID), funded by the German Ministry for Education and Research (BMBF). The experiments were conducted at the Norwegian National Mesocosm Centre, Espesgrend, in cooperation with the Department of Biology, University of Bergen.

Figure 1. Mean surface area of a) lapillus and b) sagitta (both normalized to fish standard length, SL) from Atlantic cod larvae grown at three $p\text{CO}_2$ treatments (C=370 μatm , M=1800 μatm , H=4200 μatm). Whiskers denote 95% confidence interval. Different letters above bars denote significant differences between $p\text{CO}_2$ treatments based on Analysis of Covariance test with SL as the covariate (at 5% significance level). Numbers inside bars indicate sample sizes.

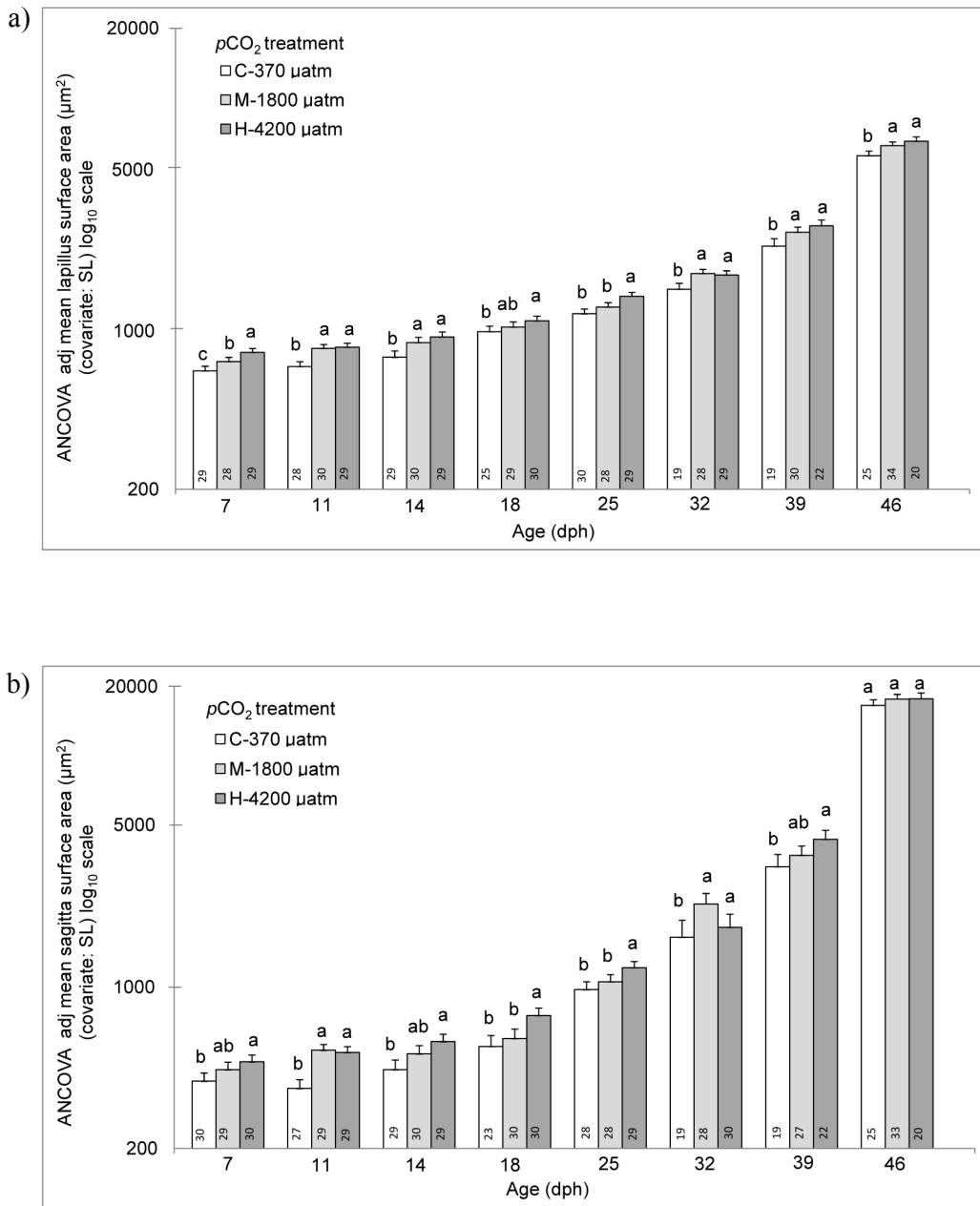


Figure 2. Sagitta/Lapillus surface area ratio of otoliths from cod larvae grown under three $p\text{CO}_2$ concentrations (Control=370 μatm , Medium=1800 μatm , High=4200 μatm). Whiskers denote 95% confidence interval. Different letters above whiskers denote significant differences between $p\text{CO}_2$ treatments based on Nested-design Analysis of Variance or Kruskal Wallis test, if necessary (at 5% significance level).

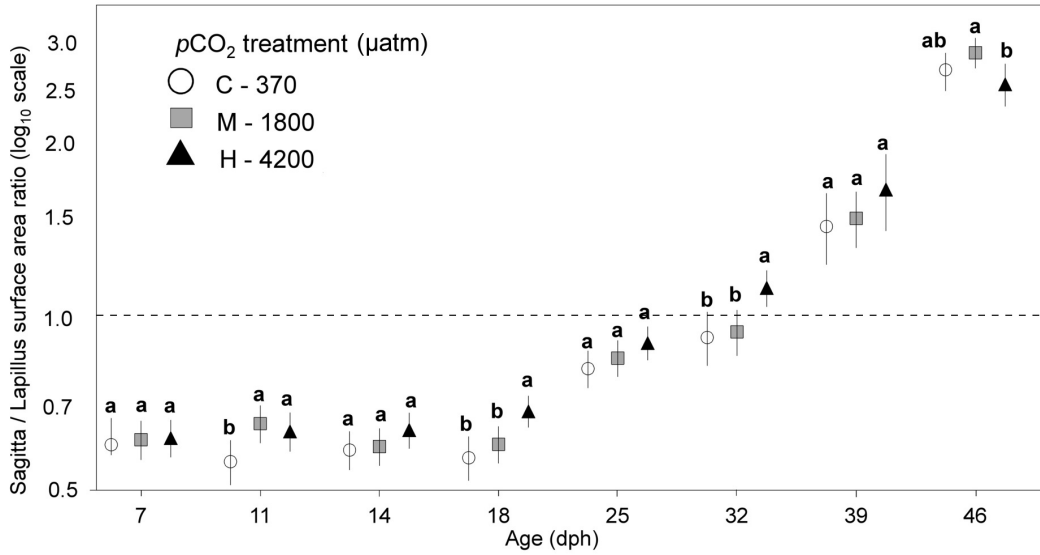


Figure 3. Standard lengths of cod larvae grown under three $p\text{CO}_2$ concentrations (Control=370 μatm , Medium=1800 μatm , High=4200 μatm). Whiskers denote 95% confidence interval. Different letters above whiskers denote significant differences between $p\text{CO}_2$ treatments based on Nested-design Analysis of Variance or Kruskal Wallis test, if necessary (at 5% significance level).

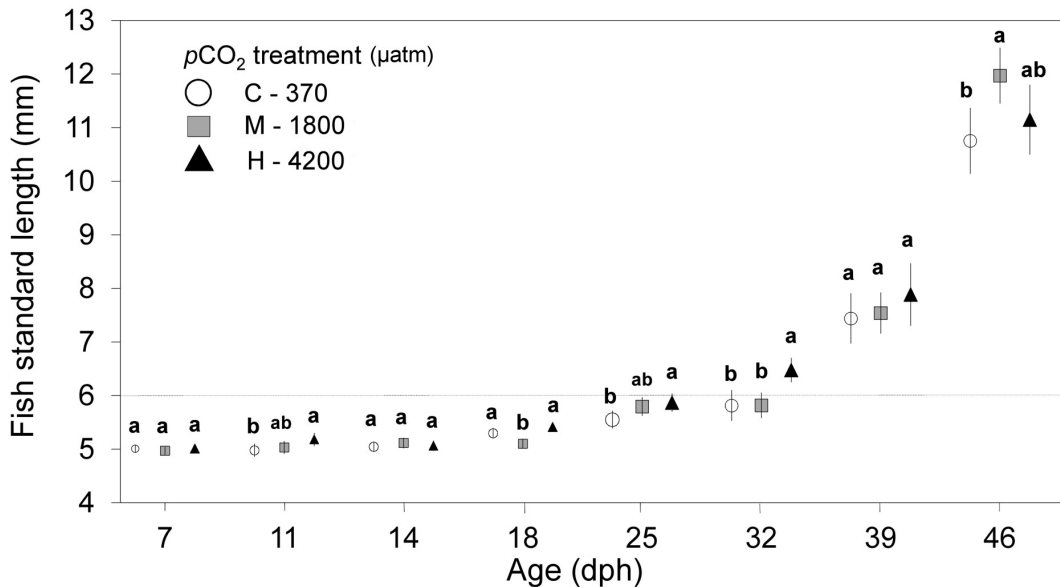


Figure 4. Scatterplots of the signed difference of otolith surface area between the right and left otoliths of Atlantic cod larvae (*Gadus morhua* L.) grown under three $p\text{CO}_2$ concentrations (Control=370 μatm , Medium=1800 μatm , High=4200 μatm). Signed difference was normalized by dividing with the mean surface area of the two sides. Offset in the x-axis was used to display the data per $p\text{CO}_2$ treatment. a) Lapillus b) Sagitta.

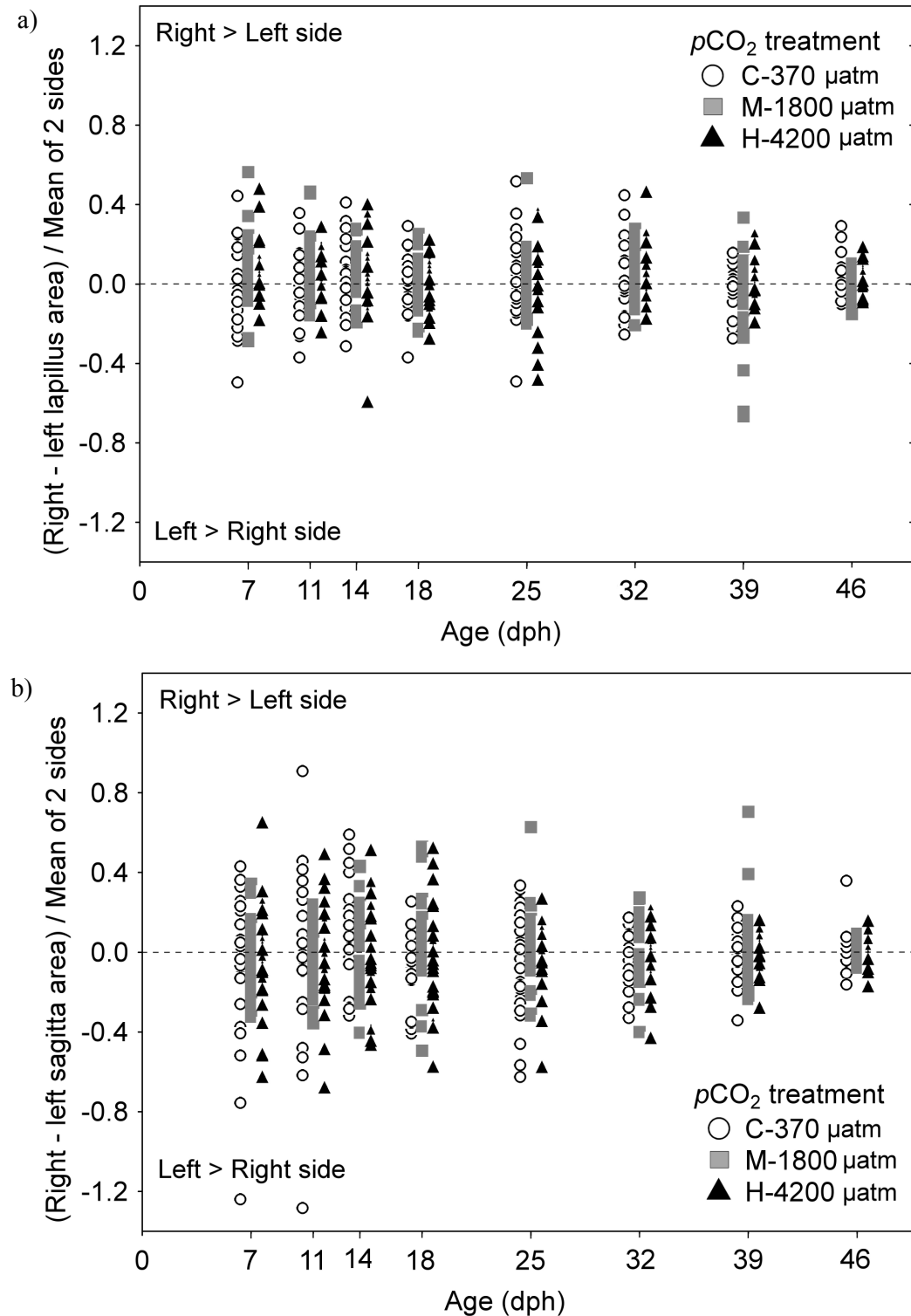
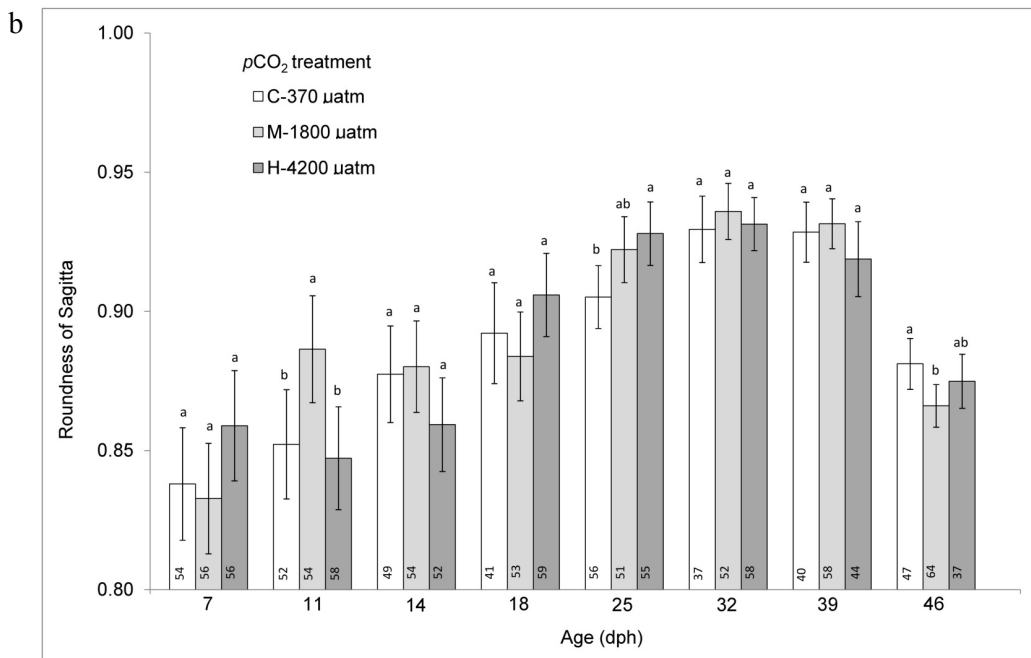
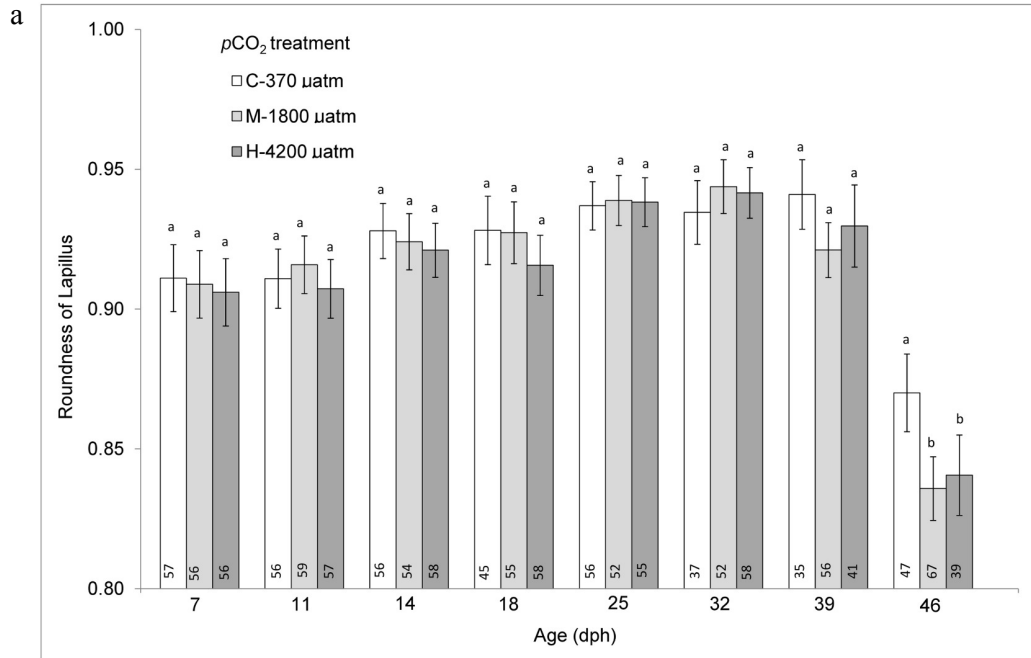


Figure 5. Mean roundness of a) lapillus and b) sagitta from cod larvae grown under three $p\text{CO}_2$ concentrations (Control=370 μatm , Medium=1800 μatm , High=4200 μatm). Whiskers denote 95% confidence interval. Different letters above whiskers denote significant differences between $p\text{CO}_2$ treatments based on Nested-design Analysis of Variance (at 5% significance level). Roundness values range from 0-highly elongated to 1-perfect circle. Numbers inside bars indicate sample sizes.



Chapter II

Chapter II

The swimming kinematics of larval Atlantic cod, *Gadus morhua* L., are resilient to elevated seawater $p\text{CO}_2$

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Abstract:

Kinematics of swimming behavior of larval Atlantic cod, aged 12 and 27-days post hatch (dph) and cultured under three $p\text{CO}_2$ conditions (control-370, medium-1800, and high-4200 μatm) from March to May 2010, were extracted from swim path recordings obtained using silhouette video photography. The swim paths were analyzed for swim duration, distance and speed, stop duration, and horizontal and vertical turn angles to determine whether elevated seawater $p\text{CO}_2$ - at beyond near-future ocean acidification levels- affects the swimming kinematics of Atlantic cod larvae. There were no significant differences in most of the variables tested: the swimming kinematics of Atlantic cod larvae at 12- and 27-dph were highly resilient to extremely elevated $p\text{CO}_2$ levels. Nonetheless, cod larvae cultured at the highest $p\text{CO}_2$ concentration displayed vertical turn angles that were more restricted (median turn angle, 15°) than larvae in the control (19°) and medium (19°) treatments at 12-dph (but not at 27-dph). Significant reduction in the stop duration of cod larvae from the high treatment (median stop duration, 0.28s) was also observed compared to the larvae from the control group (0.32s) at 27-dph (but not at 12-dph). The functional and ecological significance of these subtle differences are unclear and, therefore, require further investigation in order to determine if they are ecologically relevant or spurious.

Introduction

The high latitude regions, including the Norwegian Sea and Barents Sea, are referred to as the "bellwether" for future trends of ocean acidification (Fabry et al. 2009). These areas are sensitive to ocean acidification because of increased CO₂ solubility at low water temperatures, greater air-sea gas exchange as sea ice loss increases from global warming, and CO₂-remineralization of high organic carbon load from seasonal primary production and low alkalinity riverine inputs. According to the global coupled carbon cycle-climate model of Steinacher et al. (2009), the Arctic surface waters will experience the largest model-simulated pH changes over the 21st century with hydrogen ion concentration increases of up to 185% or an equivalent pH decline of 0.45 units in response to a global mean atmospheric CO₂ concentration of 850 ppmv under the A2 IPCC business-as-usual scenario (IPCC 2007). Assuming that the ocean and atmosphere are in equilibrium with respect to CO₂, partial pressure of CO₂ ($p\text{CO}_2$) in seawater roughly mimics that of the atmosphere (Gattuso and Lavigne 2009). Further, localized decreases in pH - in addition to these global predictions - can result from seasonal upwelling of high $p\text{CO}_2$ bottom water. Such events were recorded inside the bay and along the inner shelf of the Gulf of Alaska in September 2008 with aragonite undersaturated waters measured close to the surface until about 450 meters deep (Fabry et al. 2009). Seasonal occurrences of aragonite-undersaturated subsurface waters, resulting from anthropogenic CO₂ uptake, were reported in 2002 to 2004 in the Chukchi Sea-which, like the Barents Sea, functions as an inflow shelf to the Arctic Ocean (Bates et al. 2009). The Norwegian Sea and Barents Sea act as a carbon sink from the first part of the year until the end of summer due to the biological draw down of $p\text{CO}_2$ and cooling of surface water during transit (Findlay et al. 2008; Bates and Mathis 2009). Gislefoss et al. (1998) reported typical surface water $p\text{CO}_2$ values in the Norwegian Sea during winter at 357 μatm and summer as low as 270-300 μatm from 1991 to 1994. Thus, pelagic organisms in the Norwegian and Barents Sea might be naturally adapted to lower $p\text{CO}_2$ and high aragonite saturation during the summer period. However, how future ocean acidification events may impact these organisms is an open question.

A number of studies have reported on the detrimental effects of ocean acidification on the behavior and sensory responses of marine fishes. White seabass larvae grown at 2500 μatm were lethargic and swam less than control larvae (Huelsenbeck 2010). Damage to the olfactory ability of a clownfish, *Amphiprion percula*, resulted in a loss of homing ability to reef habitat and suitable settlement sites at

pH 7.8 and 7.6 (Munday et al. 2009b) and loss of predator detection/avoidance abilities at 700-1000 $\mu\text{atm CO}_2$ (Munday et al. 2010; Dixson et al. 2010). The brown dottyback, *Pseudochromis fuscus*, exposed to elevated $p\text{CO}_2$ exhibited a negative reaction to the smell of injured prey seemingly making them less able to respond to changing food availability (Cripps et al. 2011). Hearing ability of juvenile clownfish reared in elevated $p\text{CO}_2$ of 600 to 900 μatm was also damaged with juveniles attracted to reef noise that exposed them to greater predation risks (Simpson et al. 2011). Domenici et al. (2012) provided evidence of damage to brain function resulting from elevated $p\text{CO}_2$ by showing a reduced level of lateralization or ability to decide between left and right turns in a reef fish, *Neopomacentrus azysron*. Nilsson et al. (2012) reported that elevated $p\text{CO}_2$ affects neurotransmitter function (GABA-A receptors) in the brain of larval reef fish species resulting in seemingly maladaptive olfactory choices and loss of behavioral lateralization. The ability of damselfish, *Pomacentrus amboinensis*, to learn from chemical and visual information of a predator was also damaged at elevated $p\text{CO}_2$; non-display of anti-predatory response even with previous exposure to predator cues (Ferrari et al. 2012).

Changes in the swimming behavior of fish larvae - in response to ocean acidification, for example - are difficult to examine due to the very small size and transparency of the fish. Fish kinematics analyzes changes of position as a function of time without reference to hydrodynamic forces (Videler 1993). Fish kinematics has wide application in foraging studies such as the characterization of prey search behavior (O'Brien et al. 1989; Browman and O'Brien 1992), foraging under variable prey concentrations (Coughlin et al. 1992; Puvanendran et al. 2002) and under different spectral qualities and intensities of light (Browman et al. 1994; Vollset et al. 2011), and in escape responses from predators (Webb 1981; Domenici 2001; Skajaa and Browman 2007). Given all of the behavioral changes observed in the studies cited above, it seems reasonable to expect that elevated $p\text{CO}_2$ will also have an effect on the kinematics of larval movements.

One of the most economically important commercial fish species in the north Atlantic is the Atlantic cod, *Gadus morhua* L. The largest Atlantic cod stock is the Arcto-Norwegian cod in the Barents Sea (Suthers and Sundby 1993). Arcto-Norwegian cod and Norwegian coastal cod release pelagic eggs along a 1200-kilometer coastline from mid- to northern Norway. Eggs hatch and are advected to the Barents Sea during an approximately 100-day planktonic larval and early juvenile phase (Houde and Zastrow

1993; Suthers and Sundby 1993). The timing of release of the pelagic early life stages of these two cod stocks coincides with the seasonal surface increase of calcite and aragonite saturation states and with the draw-down of $p\text{CO}_2$ during spring and summer primary production (Findlay et al. 2008). This decreases the likelihood that early life stages of cod in this region are pre-adapted to fluctuating low pH.

Considering the economic importance of Atlantic cod, and its presence at high latitudes in waters that are sensitive to ocean acidification, this study was conducted to investigate whether elevated seawater $p\text{CO}_2$ affects the swimming kinematics of Atlantic cod larvae. $p\text{CO}_2$ values beyond near-future ocean acidification scenarios were selected to assess the possible effect of extreme acidification events such as those associated with upwelling.

Materials and methods

Seawater manipulation

Cod larvae were grown in land-based mesocosms at the University of Bergen's Espegrend Marine Station from March to May 2010 at three $p\text{CO}_2$ levels: control (370 μatm), medium (1800 μatm) and high (4200 μatm), with three replicates for each treatment level (Table 1).

The 2650-L replicate tanks of 1.5-m height were placed inside two water baths to keep the temperature relatively stable with a starting temperature of 5°C in March which rose to 10°C by May. Seawater was supplied to the tanks using a flow through system from a 40-meter deep water intake. Salinity was relatively constant at a mean of 33.3 psu and dissolved oxygen concentration remained above 90% saturation. For further details, see Frommel et al. (2012).

The targeted $p\text{CO}_2$ levels were achieved by bubbling CO_2 through diffusers to the bottom of the tanks close to the water inflow and aeration bubbles. This provided rapid CO_2 mixing in the water column and appropriate water circulation. The amount of CO_2 added to the tanks was regulated by magnetic valves using a feedback mechanism from a pH probe in each tank, which was connected to an Aquastar IKS computer. The pH probes provided continuous daily pH_{NBS} measurements of all tanks at 15-minute intervals, which were recorded by the computer. In addition, daily measurements of pH in the tanks were made with a hand-held laboratory glass pH probe (WTW) to check the pH values of the Aquastar IKS. The WTW pH probe was calibrated with seawater standard and seawater certified reference material (Oceanic Carbon Dioxide Quality

Control, Dr. Andrew G. Dickson, Scripps Institution of Oceanography). Water samples for dissolved inorganic carbon (DIC) and alkalinity were also collected weekly for the calculation of the total carbonate chemistry using CO2SYS (Lewis and Wallace 1998).

Larval rearing

Newly fertilized Norwegian coastal cod eggs were obtained from the Parisvatnet Field Station of the Institute of Marine Research on March 25, 2010. The eggs were immediately transferred to incubation buckets floating inside each replicate tank at the Espegrend mesocosm facility at an equal stocking density. After three days, the pH was adjusted to the treatment level. Dead eggs were removed from the incubation buckets until the 50% hatching day on April 9, 2010, also designated as 0-days post hatch (dph). On the same day, the newly hatched cod larvae were redistributed among the replicate tanks so that each treatment had a similar initial stocking density (ca. 4 larvae L⁻¹).

The larvae were fed with natural zooplankton. Seawater near the station was filtered daily using a Hydrotech size-selective filter system (Seljeset et al. 2010). Zooplankton of 80-250 µm (primarily copepod nauplii and rotifers) were added daily to each tank initially, and replaced gradually by zooplankton 350-500 µm (nauplii and small copepods) by 34-dph. The residual zooplankton density in each tank was monitored daily prior to feeding to achieve a prey density of 2000 prey L⁻¹ (following Puvanendran et al. 2002).

Since the rearing experiment was performed outdoors, the larvae were exposed to a natural light cycle and intensity. Average light intensities measured using a LI-COR Underwater Quantum Sensor during a sunny day and zero cloud cover were 330 and 93.6 µmol s⁻¹m⁻² at 5-cm below water surface and at bottom of tanks, respectively.

Handling of animals in the mesocosm-experiment was conducted using the animal experimentation permit ID2346 granted by the Animal Welfare Committee as determined under the Norwegian Animal Welfare Act.

Video recording of larval swimming behavior

The swimming behavior of 12 and 27-dph Atlantic cod larvae was recorded using silhouette video photography (SVP). The use of SVP to analyze the kinematics of behavior of cod larvae is a powerful technique compared to conventional video imaging tools because it allows for recording of behavior with a large depth and field, very good

resolution, and low intensity light sources to achieve the silhouette effect (see Browman et al. 2003).

During the night prior to observation and recording, around 60 larvae were randomly sampled from each replicate tank and placed in floating buckets in tanks with mesh bottoms that kept food out. Around 4 am, the sampled larvae were transferred to cylindrical transparent plastic bags with 7 liters of seawater from each tank

The plastic bags were then placed inside cooler boxes to keep water temperature stable during transport. Transport of larvae from the Espegrend mesocosm facility to the Institute of Marine Research's Austevoll Research Station took about 2 hours. Transport of cod larvae to the Austevoll Research Station was previously reported to have no detrimental effect on the larvae (Vollset et al. 2011). The larvae were kept in the dark from the time of sampling until 10 minutes prior to recording to inhibit feeding. Thus, it is assumed that during recording, the larvae were hungry and motivated to look for prey.

Approximately 50 larvae from each replicate tank were transferred into separate 20x20x20cm observation tanks inside the SVP room (in darkness). One observation tank was used for each replicate tank. Seawater from the treatment tanks was used both to transport the larvae and in the observation tank. The observation tanks were sealed with plastic to reduce efflux of CO₂. A separate test of CO₂-efflux showed that the pH and pCO₂ levels of the three treatments remained distinguishable after simulated transport conditions, although there was a slight loss of approximately 360 µatm from the highest treatment. Room temperature was maintained close to the average mesocosm seawater temperature: 6.7°C for 12-dph and 8.9°C for 27-dph. The recording sequence of replicate tanks was High-R1, Control-R3, Medium-R3, Control-R2, High-R2, Medium-R2, Medium-R1, Control-R1 and High-R3. The outer five cm of the observation tanks were covered with black plastic contact paper, which restricted the field of view to the central 15 cm volume of water. This ensured that surface or edge effects did not affect the observations. A known amount of fresh zooplankton was added to the observation tank 10 minutes prior to recording to motivate foraging behavior. The prey density during the observations was about 550 prey L⁻¹. The total recording time for each replicate was 30 minutes with temporal resolution of 25 frames per second. Complete descriptions of the observation procedures and the principles of the SVP and the analysis of swim paths is reported in Browman et al. (2003).

Analysis of swim paths

Reconstruction of the individual fish swim paths was done by combining the two orthogonally-recorded videos of each observation tank using the TrakFish software (Racca Scientific Consulting and JASCO Research Ltd., Victoria, British Columbia, Canada). In TrakFish, the two videos were calibrated by creating a reference volume from four marks with known coordinates recorded against each front of the observation tank facing the camera. The reference volume established a scaled coordinate system from which the three-dimensional spatial coordinates of fish location was derived. Swim paths were reconstructed frame-by-frame from the initial, middle and last five minutes of the observation to provide representative sampling.

Using the Anapaths software (also from Racca Scientific Consulting and JASCO Research Ltd., Victoria, British Columbia, Canada), paths that were closely adjacent to each other and seemed to resemble a single swim path were joined together. Paths that were too short (below 2 body lengths) and/or had extremely jagged (unrealistic) swim trajectories were not included in the analysis. The kinematic variables of the swim paths namely, move duration, distance and speed, stop duration, and horizontal and vertical turn angles (i.e. change in direction after a stop in a horizontal and vertical planes) were extracted using the Anapaths software. Every data point from each reconstructed fish swim path was used as an observation value for statistical analysis. The output files for each replicate tank consisted of a list of recorded - for example - turn angles, which did not differentiate individual fish in the tank.

Data analysis

The horizontal and vertical turn angle components of the swim paths were analyzed using circular statistics (Batschelet 1981). A non-parametric Mardia-Watson Wheeler test (MWW test) was used to compare distributions of the turn angles between replicates within each treatment, and then between treatments provided that replicates did not show any significant differences. If data showed higher variability between replicates than between treatments, it was evident that treatments had no effect.

For the other variables (stop duration, move duration, move length, and move speed), a non-parametric two-sample Kolmogorov-Smirnov (KS) test was used to compare the distribution of replicates within treatments and then between treatments, again, provided that replicates did not show any significant differences. When a

significant difference was found a Mann-Whitney test was applied to compare the medians between treatments. A 5% significance level was used in all tests.

Results

Age-related differences: Age-related increase in horizontal turn angle was detected (MWW, $p < 0.001$). Median turn angles for 12 and 27-dph larvae were respectively 33° and 38° . Move duration was statistically different between ages (Mann-Whitney test, $p < 0.05$). Older larvae had a higher count of short move durations (25% percentile was slightly higher in 27-dph larvae).

Turn angles: Turn angles were highly variable between replicates for both age groups and confidence intervals were generally overlapping (Figure 1,2). Only the vertical turn angle at 12-dph had all treatments with homogenous replicates and a statistically significant difference was found between the high treatment (median turn angle, 15°) and the medium or control treatments (both at 19°) (MWW test $p_{C-H} = 0.006$, $p_{M-H} < 0.001$) (Figure 1a, Table 2). Vertical turn angles for high $p\text{CO}_2$ -treated fish (4200 μatm) were 21% narrower than for control and medium cod larvae (Figure 1a). There was no significant difference between medium and control treatments ($p > 0.05$).

Linear variables: The only difference in treatments that was statistically significant, both in distribution and in median, was in 27-dph fish. Individuals in the high treatment showed significantly shorter stop durations than fish in the control treatment (KS test: $p < 0.001$; Mann-Whitney U test: $p < < 0.001$) (Figure 3b, Table 2). This represents a 12.5% decrease in stop duration relative to the control group. Assuming a seven-hour daily "active feeding" period, the median stop durations of the control (0.32s) and high (0.28s) treatments were translated into 421.1 and 419.4 minutes of total daily search time for each treatment, respectively. Since cod larvae are saltatory searchers,- i.e. they search for prey only while stationary (Galbraith et al. 2004; Hunt von Herbing et al. 2001; Ruzicka and Gallager 2006),- the total daily search time was calculated based on the total number of stops in a given seven-hour period and the median duration of the stops for each treatment. Thus, a difference in total daily search time between the control and high treatments was estimated to be 100.8s.

Stop and move durations (median values respectively, 0.28s and 0.32s) at 12-dph did not vary between control and high treatments (KS test: $p > 0.05$) (Figures 3a, 4a,

Table 2). At 27-dph, pairwise comparison of medium treatment (median- move duration 0.32s; speed 5.9 mm s⁻¹) vs. high treatment (median, 0.36s) for move duration (Figure 4b) and vs. the control group for move speed (median, 6.0 mm s⁻¹) (Figure 5b) did not reveal significant differences (KS test: $p > 0.05$).

Treatment comparisons were not carried out for move speed at 12-dph and move distance in both age groups (Figure 5a, 6, Table 2). In such cases, at least two treatments had high variability among replicates.

Discussion

There were clear between-age differences in horizontal turn angle and move duration. However, only cod larvae from the high $p\text{CO}_2$ group showed significant changes in swimming kinematics relative to the control group. Larvae from the high $p\text{CO}_2$ treatment exhibited smaller vertical displacements at 12-dph and shorter stop durations at 27-dph.

Although the functional significance of the statistically significant differences in vertical displacement and stop duration is unclear, it is instructive to examine these changes in the context of existing explanatory frameworks for the mechanisms that modulate repositioning movements of zooplanktivorous fishes. Cod larvae are characterized as saltatory searchers (Hunt von Herbing et al. 2001; Galbraith 2004; Ruzicka 2004). O'Brien et al. (1989) coined this term to describe a stop and go swim pattern and demonstrated that the fish searched for prey only during the brief stationary periods. The relative durations of repositioning movements and pauses are modulated by environmental conditions such as light level and prey size and/or abundance. Modulation of these kinematic variables results in larvae increasing prey encounter rate when food abundance is low (or the prey are very small) and maintain position within a small area when food is abundant (area-restricted search; see Coughlin et al. 1992). It also has significant implications for energy conservation because foraging activity accounts for up to 80% of routine metabolism in cod larvae (Ruzicka 2004). The reduction in stop duration (i.e. the prey location phase) at 27-dph could mean that the larvae were spending less time looking for food. We calculated the median stop durations in this study (control=0.32s and high=0.28s) and comparing to other studies they were relatively shorter than those reported earlier for cod larvae: Ruzicka (2004), 0.78s for 27-dph; and Vollset et al. (2011), 1.64s for 26-dph larvae observed at dusk. On the other hand, the mean stop durations reported here were closer to those obtained for 6-dph cod

larvae, which had 0.3s mean stop duration (Browman et al. 2003). Roughly estimating, the difference in the median stop durations between the two treatments correspond to a reduction of 100.8s in total daily search time.

Skiftesvik et al. (2003) reported significant differences on the vertical turn angles of 5-dph cod larvae feeding on rotifers only versus larvae feeding on rotifers with algae. Larvae feeding on rotifers and algae had greater mean vertical repositioning angles. Browman et al. (2003) also reported an effect of maternal condition on the vertical repositioning angles of 6-dph cod larvae, with wider angles in larvae from well-fed mothers. Although the implications of these observations were not discussed, it is possible that, in both cases, the high concentration and contrast of prey at the edges of the search volume caused the larvae to modulate their movements by turning wider horizontally and vertically. For a wedge-shaped search volume with only a 10° vertical half-angle, prey below and above this field of view are high enough to cause the larvae to either move up or down. The current study reported restrictions in the vertical turn angle of the high- $p\text{CO}_2$ cod larvae at 12-dph; however, we can only speculate about the biological significance of this observation.

The 12 and 27-dph cod larvae used in this experiment did not show significant differences in dry weight, growth rate, or biochemical indices such as RNA/DNA ratio and protein content between $p\text{CO}_2$ treatments (Frommel et al. 2012). Only subtle changes in the swimming behavior of cod larvae were discernible in this study, even at very high concentrations of $p\text{CO}_2$. A possible reason could be that mortality during the experiment had removed individuals most sensitive to elevated $p\text{CO}_2$. Frommel et al. (2012) reported significant histological damage in the liver, pancreas, kidney, eye and the gut of cod larvae from the medium and high treatments of this experiment at 32-dph. The tissue damage was not present in the 46-dph cod larvae suggesting death of tissue-damaged individuals. The impacts of elevated $p\text{CO}_2$ can be reduced when food supply is high, for example, in blue mussels (Melzner et al. 2011) and the coral *Porites* spp. (Edmunds 2011). Therefore, it is also possible that the prey density (2000 prey L^{-1}) used in our study, which is much higher than those experienced by cod larvae in the field (Tilseth 1984; Ellertsen et al. 1984; Ellertsen et al. 1987), modulated the impacts of elevated $p\text{CO}_2$.

Our results indicate that the swimming behavior of Atlantic cod larvae is resilient to elevated $p\text{CO}_2$. Even at extremely high $p\text{CO}_2$ levels of 4200 μatm , only minor changes were reported in only two of the 12 swimming kinematic variables observed in 12- and

27-dph cod larvae. The minor changes observed in stop duration and vertical turn angles could subtly affect the ability of Atlantic cod larvae to modulate their foraging behavior, but they could also represent a statistically significant result that has no ecological significance. Our results support the previous findings by Melzner et al. (2009a) that Atlantic cod juveniles reared for 4 and 12 months in 3000 and 6000 μatm of $p\text{CO}_2$, respectively, were not compromised in their locomotory performance. It remains to be seen whether the swimming behavior of Atlantic cod larvae is affected when elevated $p\text{CO}_2$ is coupled with other stressors such as increased temperature, hypoxia and low food availability (Pörtner et al. 2005; Munday et al. 2009a; Kristiansen et al. 2011; Nowicki et al. 2012).

Acknowledgements

Funding support was provided through the European Marie Curie Initial Training Network "Calcification by Marine Organisms" (CalMarO) and the European Community's Seventh Framework Programme (FP7/2007-2013) "European Project on Ocean Acidification" (EPOCA, grant agreement N211384). The study was also supported by the project "Biological Impacts of Ocean ACIDification" (BIOACID), funded by the German Ministry for Education and Research (BMBF), and by the Norwegian Institute of Marine Research ("Fine-scale behavioral interactions in the plankton" and "Biological effects of ocean acidification" projects to HIB). The experiments were conducted at the Norwegian National Mesocosm Centre, Espesrend, in cooperation with the Department of Biology, University of Bergen and at the Institute of Marine Research's Austevoll Research Station.

Table 1. Carbonate system variables measured weekly for eight weeks for the ocean acidification experiment with larval Atlantic cod (*Gadus morhua* L.). Mean values with standard deviation: C_T , total dissolved inorganic carbon; A_T , total alkalinity; pCO_2 , partial pressure of CO_2 ; pH_T (total scale) at a salinity of 33.3.

| Treatment | Replicate | Temp. (°C) | C_T ($\mu\text{mol kg}^{-1}$ SW) | A_T ($\mu\text{mol kg}^{-1}$ SW) | pCO_2 (μatm) | pH_T |
|------------|-----------|---------------|---|---|--------------------------------|-----------------|
| Control, C | 1 | 7.2 ± 0.9 | 2103 ± 39 | 2271 ± 41 | 368 ± 78 | 8.08 ± 0.09 |
| | 2 | 7.2 ± 0.8 | 2107 ± 35 | 2283 ± 36 | 373 ± 83 | 8.07 ± 0.09 |
| | 3 | 7.2 ± 0.8 | 2110 ± 36 | 2291 ± 27 | 359 ± 70 | 8.09 ± 0.07 |
| Medium, M | 1 | 7.1 ± 0.8 | 2340 ± 40 | 2296 ± 23 | 1866 ± 559 | 7.43 ± 0.12 |
| | 2 | 7.2 ± 0.9 | 2319 ± 37 | 2283 ± 37 | 1810 ± 342 | 7.44 ± 0.09 |
| | 3 | 7.2 ± 0.8 | 2332 ± 77 | 2286 ± 46 | 1957 ± 1019 | 7.46 ± 0.22 |
| High, H | 1 | 7.1 ± 0.8 | 2461 ± 35 | 2284 ± 34 | 4170 ± 861 | 7.08 ± 0.10 |
| | 2 | 7.1 ± 0.7 | 2490 ± 49 | 2293 ± 30 | 4547 ± 872 | 7.05 ± 0.08 |
| | 3 | 7.2 ± 0.7 | 2464 ± 60 | 2296 ± 30 | 4026 ± 898 | 7.10 ± 0.10 |

Table 2. Summary of statistics. * Significant difference, NS: non-significant difference, NT: no tests were performed because replicates were significantly different within treatments. Non-parametric tests were: Mardia-Watson Wheeler test (MWW test) for turn angles; Kolmogorov-Smirnov test and Mann-Whitney test for linear parameters.

| Swim kinematics | Age (dph) | Treatment | N | Median | Min-max | Statistical significance : between replicates | Statistical significance : between treatments |
|-----------------|-----------|-----------|------|--------|---------|---|---|
| VTA | 12 | C | 618 | 19° | 0-171° | NS | C≠H |
| | | M | 673 | 19° | 0-145° | NS | M≠H |
| | | H | 1323 | 15° | 0-126° | NS | C=M |
| | 27 | C | 2268 | 15° | 0-159° | * | NT |
| | | M | 2595 | 16° | 0-175° | * | |
| | | H | 2589 | 18° | 0-154° | NS | |
| HTA | 12 | C | 350 | 36° | 0-179° | NS | NT |
| | | M | 408 | 34° | 0-174° | * | |
| | | H | 1000 | 31° | 0-177° | * | |
| | 27 | C | 1742 | 40° | 0-180° | * | NT |
| | | M | 2165 | 37° | 0-180° | * | |
| | | H | 2185 | 38° | 0-179° | NS | |
| SD | 12 | C | 704 | 0.28 | 0.1-3.3 | NS | C=H |
| | | M | 782 | 0.32 | 0.1-5.5 | * | |
| | | H | 1473 | 0.28 | 0.1-3.0 | NS | |
| | 27 | C | 2572 | 0.32 | 0.1-7.6 | NS | C≠H |
| | | M | 2837 | 0.28 | 0.1-3.6 | * | |
| | | H | 2821 | 0.28 | 0.1-3.2 | NS | |
| MD | 12 | C | 763 | 0.32 | 0.1-3.4 | NS | C=H |
| | | M | 842 | 0.28 | 0.1-3.5 | * | |
| | | H | 1583 | 0.32 | 0.1-5.1 | NS | |
| | 27 | C | 2700 | 0.32 | 0.1-3.2 | * | M=H |
| | | M | 3012 | 0.32 | 0.1-4.2 | NS | |
| | | H | 2978 | 0.36 | 0.1-2.8 | NS | |
| MS | 12 | C | 753 | 5.9 | 2.5-20 | * | NT |
| | | M | 828 | 6.0 | 3.2-20 | * | |
| | | H | 1565 | 5.9 | 2.7-20 | * | |
| | 27 | C | 2671 | 6.0 | 2.6-20 | NS | C=M |
| | | M | 2997 | 5.9 | 2.6-19 | NS | |
| | | H | 2958 | 6.0 | 2.5-20 | * | |
| ML | 12 | C | 755 | 2.0 | 0.30-19 | * | NT |
| | | M | 831 | 1.8 | 0.36-19 | * | |
| | | H | 1573 | 2.1 | 0.36-19 | NS | |
| | 27 | C | 2697 | 1.9 | 0.30-20 | * | NT |
| | | M | 3002 | 2.2 | 0.32-20 | NS | |
| | | H | 2970 | 2.2 | 0.36-20 | * | |

Figure 1. Vertical turn angles of (a) 12-dph and (b) 27-dph Atlantic cod larvae (*Gadus morhua* L.) from three $p\text{CO}_2$ levels (C-380 μatm , M-1800 μatm , H-4200 μatm). Black lines from the center denote mean vector angle and arcs at the end of the lines represent 95% confidence interval for each $p\text{CO}_2$ treatment.

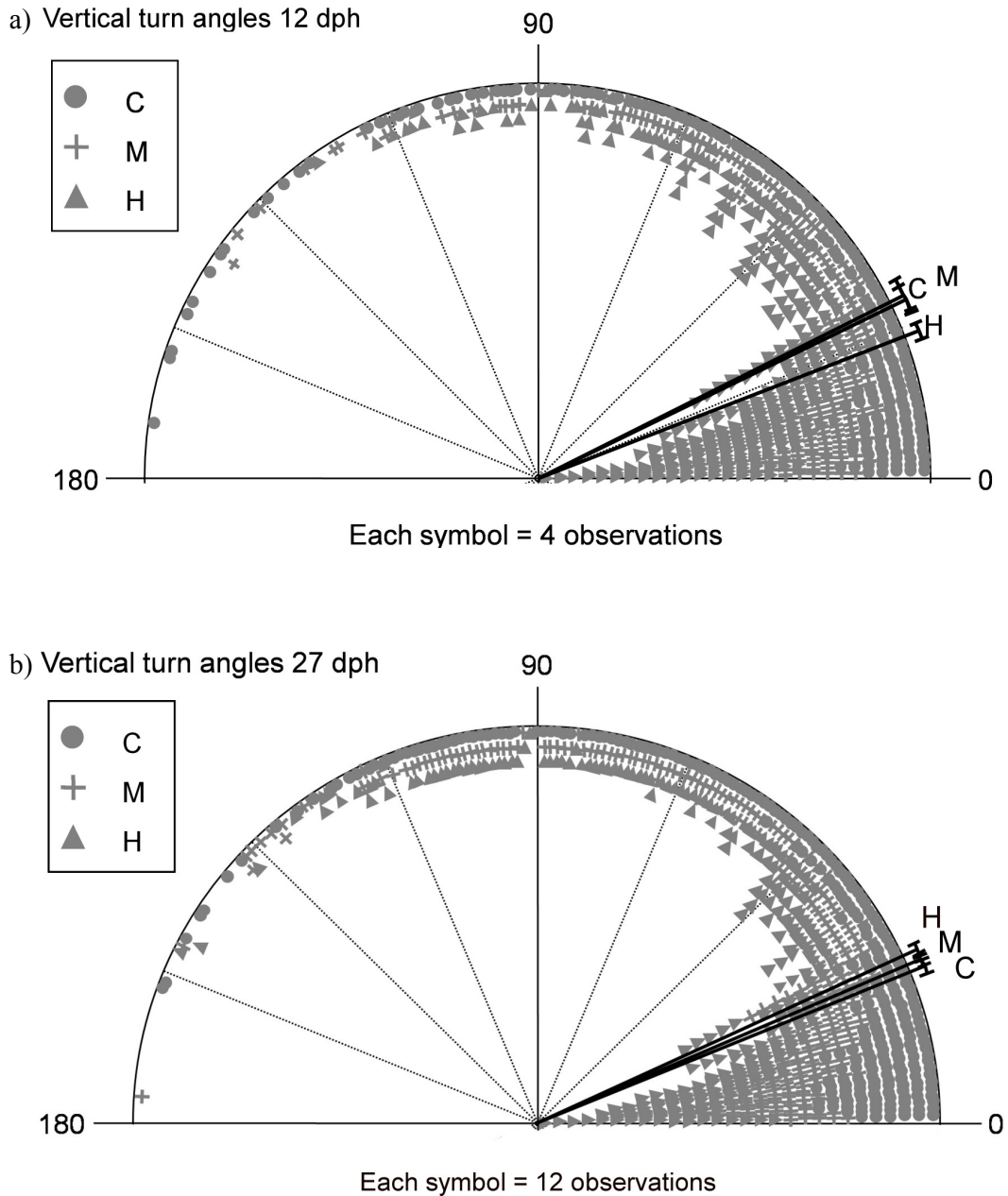
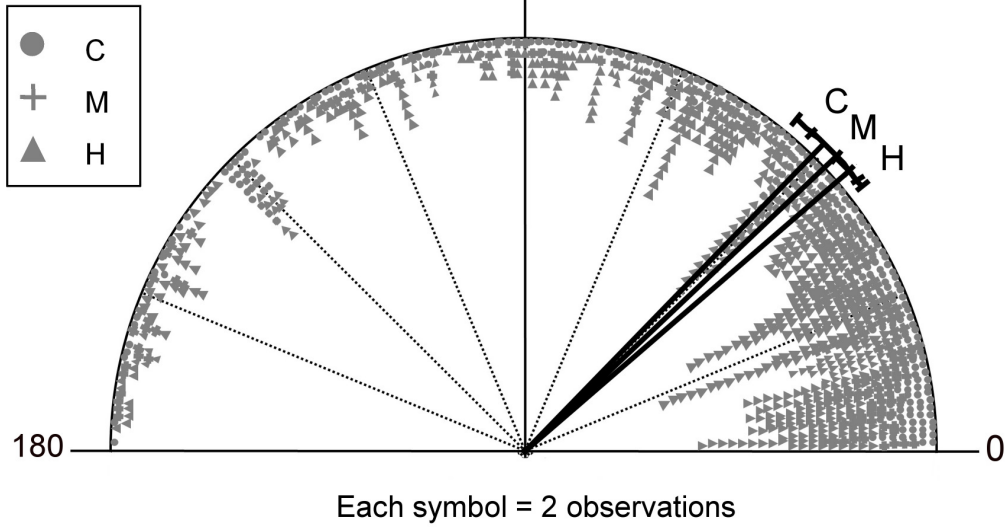


Figure 2. Horizontal turn angles of (a) 12-dph and (b) 27-dph Atlantic cod larvae (*Gadus morhua* L.) from three $p\text{CO}_2$ levels (C-380 μatm , M-1800 μatm , H-4200 μatm). Black lines from the center denote mean vector angle and arcs at the end of the lines represent 95% confidence interval for each $p\text{CO}_2$ treatment.

a) Horizontal turn angles 12 dph



b) Horizontal turn angles 27 dph

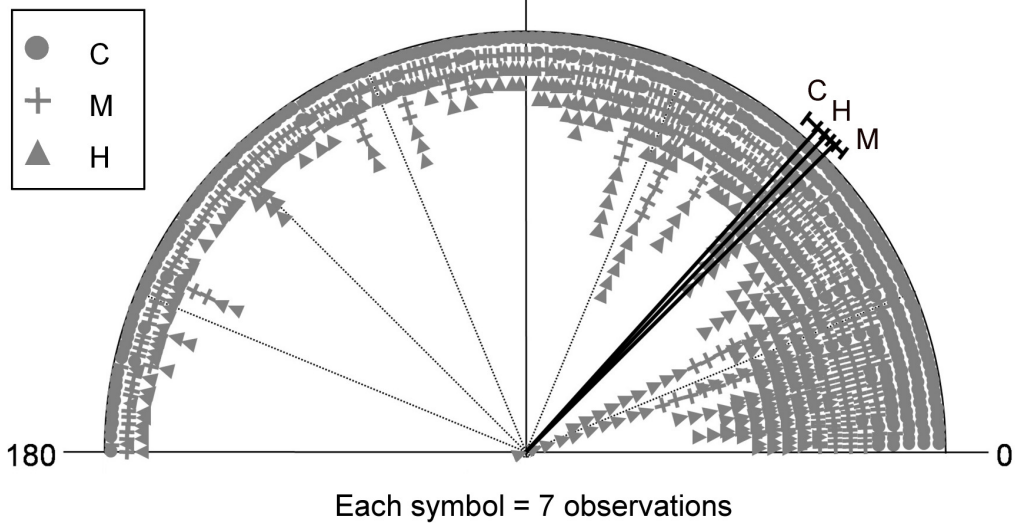


Figure 3. Cumulative fraction plots for the Kolmogorov-Smirnov tests of stop duration of Atlantic cod larvae (*Gadus morhua* L.) at (a) 12-dph and (b) 27-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Within-treatment variability in the M-group prevented comparison with other treatments.

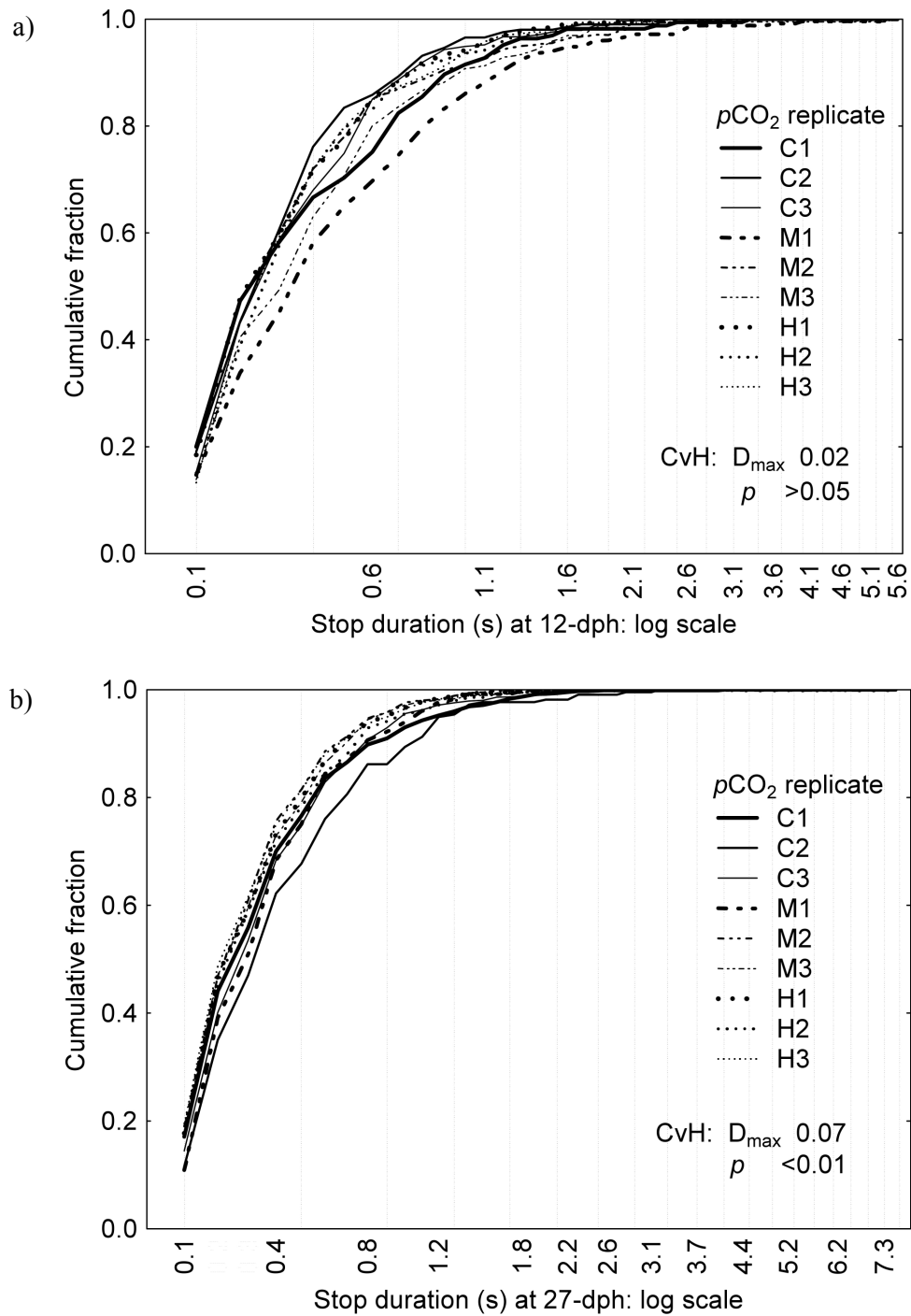


Figure 4. Cumulative fraction plots for the Kolmogorov-Smirnov tests of move duration of Atlantic cod larvae (*Gadus morhua* L.) at (a) 12 and (b) 27-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Within-treatment variability in the M and C-group at 12 and 27-dph, respectively, prevented comparison with other treatments.

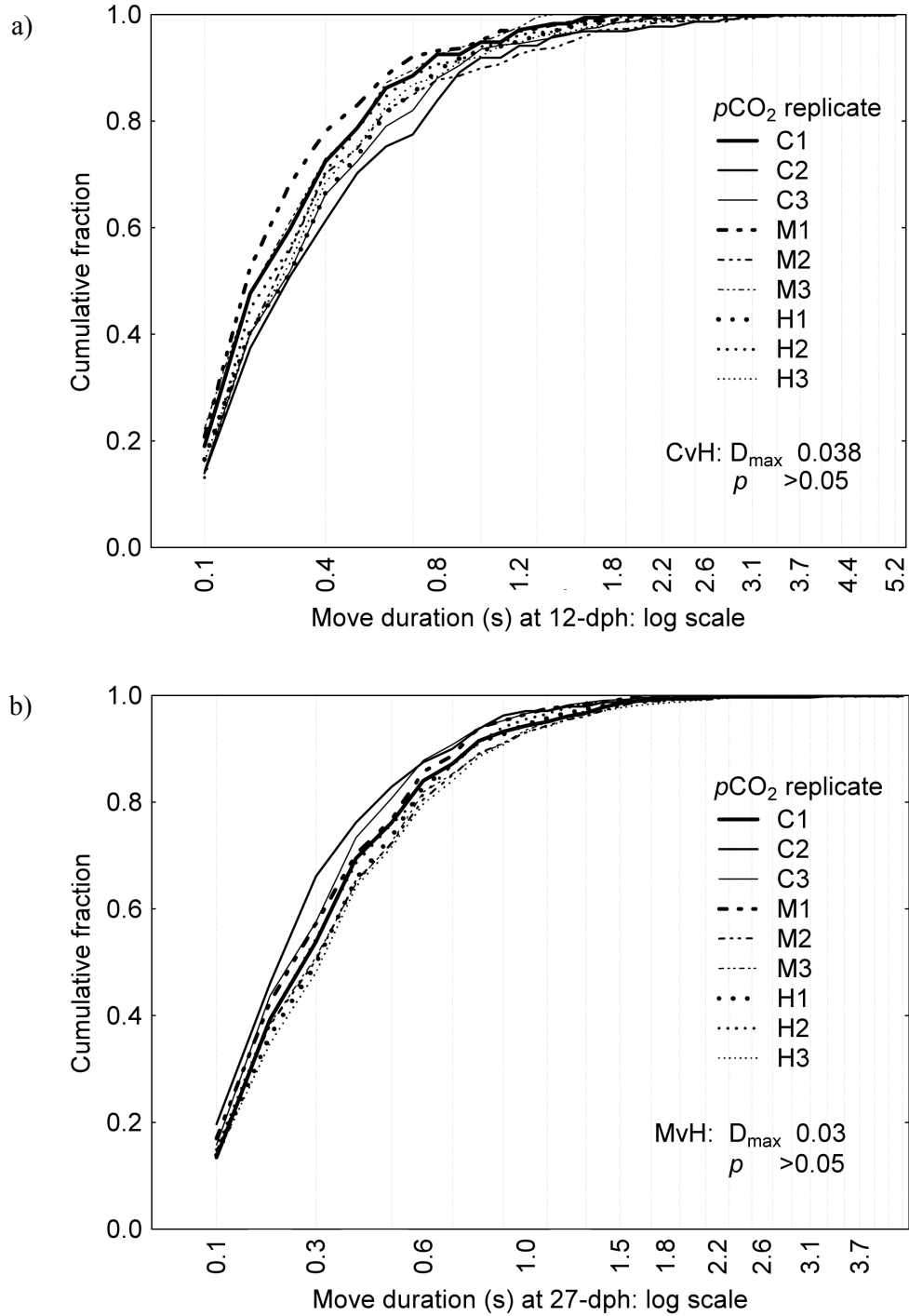


Figure 5. Cumulative fraction plots for the Kolmogorov-Smirnov tests of swim speed of Atlantic cod larvae (*Gadus morhua* L.) at (a) 12 and (b) 27-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Within-treatment variability in all the groups at 12-dph and H-group at 27-dph prevented pairwise treatment comparison.

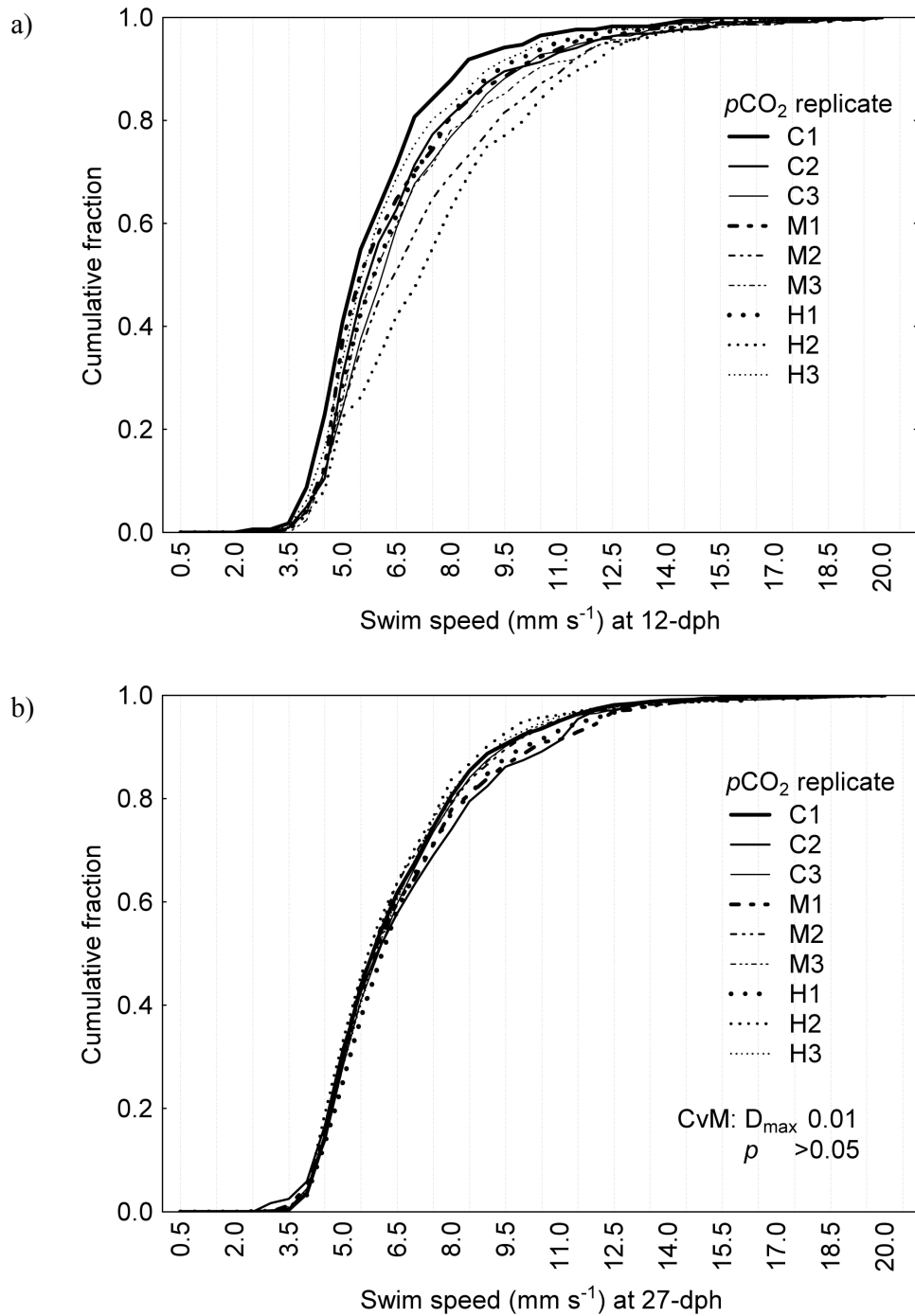
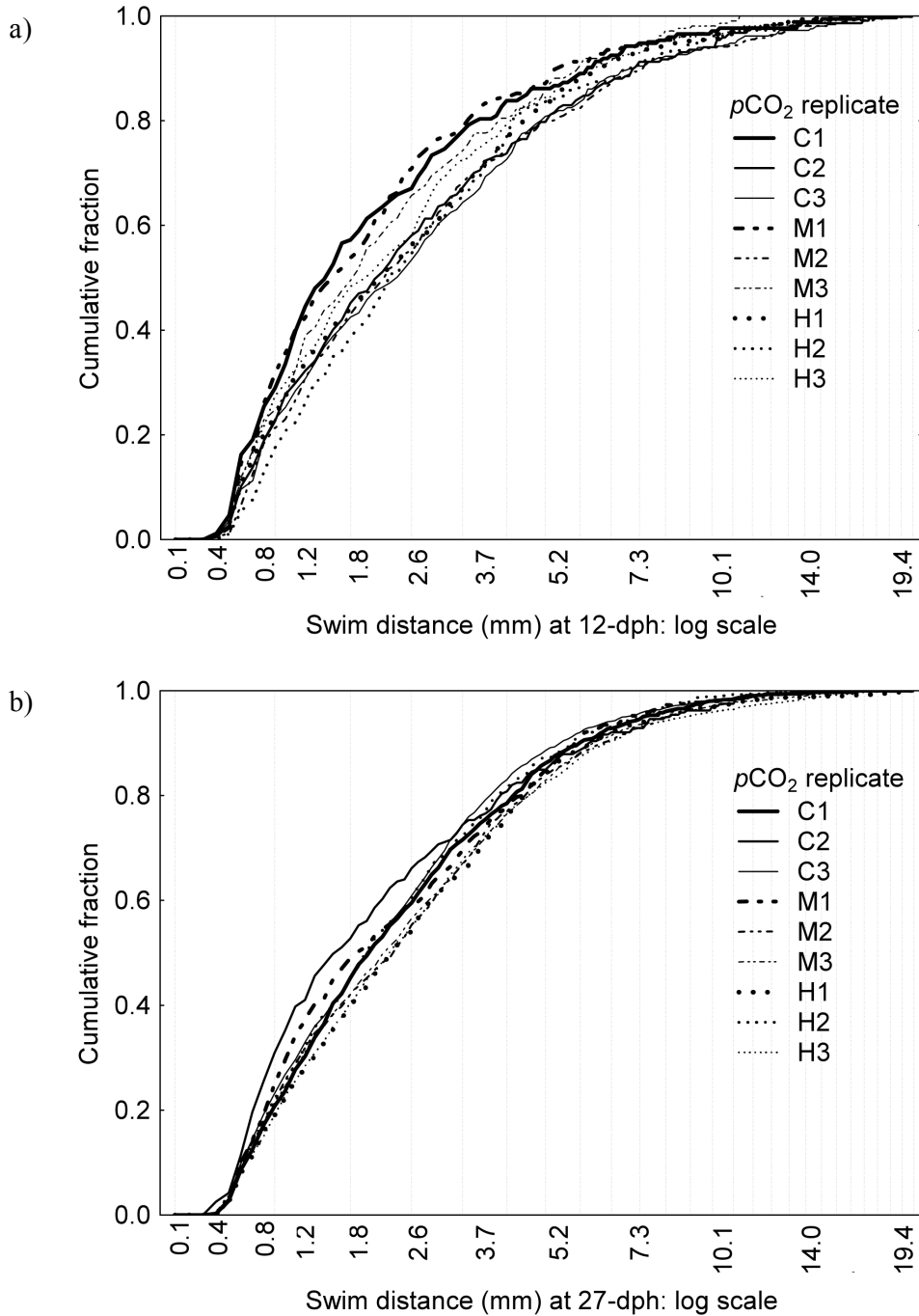


Figure 6. Cumulative fraction plots for the Kolmogorov-Smirnov tests of swim distance of Atlantic cod larvae (*Gadus morhua* L.) at (a) 12 and (b) 27-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Within-treatment variability in the C and M-group at 12-dph and C and H-groups at 27-dph prevented pairwise treatment comparison.



Chapter III

Chapter III

Effects of elevated $p\text{CO}_2$ on the swimming kinematics and foraging behavior of larval Atlantic herring, *Clupea harengus* L.

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Abstract

Kinematics of swimming behavior of Atlantic herring larvae (34 and 40-dph) grown under three $p\text{CO}_2$ conditions (control-370, medium-1800, and high-4200 μatm) from March to May 2010, were extracted from swim path recordings obtained using silhouette video photography. The swim paths were analyzed for swimming and stop duration, swimming distance and speed, and horizontal and vertical turn angles to determine the effects of elevated $p\text{CO}_2$. Most of the measures of swimming kinematics were robust to elevated $p\text{CO}_2$, but larvae from the medium treatment paused for significantly longer time (75th percentile, 0.4s) than those in the control group (75th percentile, 0.2s) at 40-dph. Observations of the foraging behavior showed that herring larvae in the high $p\text{CO}_2$ treatment had significantly more abandoned feeding strikes than those in the control group at 34-dph. However, performance of complete feeding strikes did not differ significantly among the treatments. This study showed that the swimming behavior of herring larvae observed at 34 and 40-dph were resilient to increase in seawater $p\text{CO}_2$ concentration with subtle changes observed in stop duration and abandoned feeding strikes.

Introduction

Mortality in marine fish larvae is quite high with a seven-fold magnitude decrease in abundance from eggs up to the juvenile stages (Fuiman and Werner 2002). Major processes acting on the early life stages of fish include predation, starvation, diseases, and physical environmental processes. As fish larvae grow, sensory and swimming abilities develop allowing the larvae increased ability to avoid and evade predators (Fuiman 1989) as well as enhanced prey location and capture (Haus and Peck 2009). However, development of sensory abilities may lag behind the development of the swimming apparatus of fish larvae. For example, the response of herring larvae to predator attacks was constantly low until larval length of 26 mm, beyond which responsiveness increased rapidly brought about by the late development of functional auditory bullae (Fuiman 1989). This puts emphasis on having good swimming abilities at early larval stages to compensate for inadequate sensory abilities.

Interspecific differences cause fish larvae to employ specific foraging strategy, which could either be ambush, pause-and-go (saltatory), or cruise strategy (Browman and O'Brien 1992). We have previously demonstrated that elevated $p\text{CO}_2$ (up to 4200 μatm) resulted in subtle changes in the swimming kinematics of Atlantic cod larvae, a saltatory forager, causing shorter stop duration and more restricted vertical turn angles (Maneja et al. 2012). The decrease in stop duration might affect the ability of cod larvae to look for prey since the time for visual prey search is spent when the larva is not actively swimming. On the other hand, restricted vertical turn angle could decrease the larva's ability for rapid displacement along the body's vertical axis. However, this study also showed that most of the swimming kinematics variables of cod larvae such as swimming duration, distance and speed, and horizontal turn angle were quite robust to increase in levels of $p\text{CO}_2$. Thus, species-specific swimming strategies between cod and herring larvae might elicit different reactions to increase in $p\text{CO}_2$. Nonetheless, both species can modify their swimming behavior, for example, in response to variation in prey availability, light and turbulence (Batty 1987; Ruzicka 2004; Munk and Kiørboe 1985; MacKenzie and Kiørboe 1995). Thus, changes to any of the swimming kinematic variables could affect the swimming and foraging behavior of fish larvae, which in turn could affect their survival.

Atlantic herring is highly plastic with wide range of discrete spawning seasons, and eggs and larvae adapted to wide seasonal conditions of temperature, light, hydrographic conditions, predator fields, and food availability (Geffen 2009). However,

it could also make certain herring populations more vulnerable to ocean acidification especially those spawned in well-ventilated and highly-flushed benthic environments but are at higher risk of anthropogenic $p\text{CO}_2$ encroachment in the near future. Adaptations to well-ventilated benthic environments had evolved less-developed circulatory and vascular systems, which are vital for efficient acid-base regulation, in early herring larval stages prior to metamorphosis (Soin 1971). Shallowing of the aragonite saturation horizon and decrease in surface pH (Olafsson et al. 2009, Gangstø et al. 2008, Steinacher et al. 2009) could expose the eggs in some benthic spawning grounds and pelagic larvae of Atlantic herring to high $p\text{CO}_2$ conditions.

In this study, we investigated the effects of elevated $p\text{CO}_2$ on the swimming and foraging behavior of Atlantic herring larvae.

Materials and methods

Seawater manipulation

A mesocosm experiment with three $p\text{CO}_2$ levels (control: 370 μatm , medium: 1800 μatm , high: 4200 μatm) and three replicates was conducted in the land-based mesocosms at the University of Bergen's Espegrend Marine Station from March to May 2010. A flow-through system supplied seawater pumped from a 40-meter deep water intake to the 2650-L, 1.5-m depth tanks. CO_2 was introduced using computer controls into the bottom of the tanks by bubbling through diffusers. The diffusers were placed close to the water inflow and aeration bubbles in order to facilitate efficient CO_2 diffusion and water circulation throughout the water column. Water temperature was kept relatively stable by placing the replicate tanks inside two water baths. Water temperature was about 5°C in March and increased to 10°C by the end of May. Dissolved oxygen concentration was above 90% saturation and salinity was constant at a mean of 33.3 psu. For further details, see Frommel et al. (2012).

Larval rearing

Adult, ripe herring from Lindåspollene in western Norway were strip-spawned onto glass plates and suspended into the middle of each tank on March 24, 2010. To avoid possible parental effects, fertilized eggs from each pair of male and female herring were distributed in all the tanks. The pH of the tanks was adjusted to the target treatment pH levels at four days post fertilization. Prior to the start of hatching, the suspended glass plates were placed inside floating buckets in order to collect the newly-hatched larvae.

At 50%-hatching day on April 16, 2010 (0-day post hatch, dph), hatched larvae from the same treatment were collected and redistributed to the replicate tanks in order to have a similar stocking density.

Fresh natural zooplankton was filtered daily from the adjacent seawater using a Hydrotech size-selective filter system to provide a feeding density of 2000 prey L⁻¹ (Seljeset et al. 2010). Larvae were fed initially with 80-250 µm-sized zooplankton, which was gradually increased to 350-500 µm by 27-dph.

The larvae were exposed to natural light cycle and light intensity. During a sunny day and zero cloud cover, average light intensities were 330 and 93.6 µmol s⁻¹m⁻² at 5-cm below water surface and at bottom of tanks, respectively

Handling of animals in the mesocosm-experiment was conducted using the animal experimentation permit ID2346 granted by the Animal Welfare Committee as determined under the Norwegian Animal Welfare Act.

Video recording of larval swimming behavior

A 3-dimensional video recording system, silhouette video photography, was used to observe the swimming behavior of 34 and 40-dph Atlantic herring larvae (Browman et al. 2003). Approximately 60 larvae per tank were caught and placed in floating buckets inside each tank the night prior to video recording. The floating buckets had a mesh bottom to keep live zooplankton out and provide adequate water exchange.

Around 4 am the following day, cylindrical transparent plastic bags containing seven liters of seawater from each tank were used to transport the larvae to the Institute of Marine Research's Austevoll Research Station. To keep the water temperature stable, the plastic bags were placed inside cooler boxes. The larvae were not fed and kept in the dark until the video recording as a motivation to forage for food during the observation period. Vollset et al. (2011) had previously reported that transport of fish larvae from Espegrend to Austevoll Research station did not have detrimental effects on the larvae.

The seawater containing the herring larvae was carefully transferred into separate 20x20x20-cm observation tanks inside the dark, temperature-controlled SVP room. Air temperature inside the room was adjusted to match the water temperature in the mesocosm tanks. The observation tanks were sealed with plastic covers, which were tightly secured with elastic bands, to reduce efflux of CO₂. Diffusion of CO₂ out from the seawater was previously estimated to be around 360 µatm only from the highest pCO₂

treatment and did not result in the convergence of pH and $p\text{CO}_2$ levels after eight hours without CO_2 -bubbling (Maneja et al. 2012).

The recording sequence of replicate tanks was High-R1, Control-R3, Medium-R3, Control-R2, High-R2, Medium-R2, Medium-R1, Control-R1 and High-R3. Surface or edge effect on the swimming behavior of recorded larvae was avoided by covering the outer five cm of the observation tanks. Ten minutes prior to recording, fresh zooplankton was added into the tanks in order to initiate feeding by the larvae. The recording duration for each replicate was 30 minutes with temporal resolution of 25 frames per second (Browman et al. 2003).

Growth parameter (i.e. standard length) of herring larvae used here were extrapolated from separate sampling periods conducted eight times from 0 to 39-dph. Representative sampling throughout the water column was carried out by dropping a PVC-pipe with a closing valve at the end. A calibrated picture for each larva were taken and later used to measure the standard length.

Analysis of swim paths

Reconstruction of the individual fish swim paths was done by combining the two orthogonally-recorded videos of each observation tank using the TrakFish software (Racca Scientific Consulting and JASCO Research Ltd., Victoria, British Columbia, Canada). In TrakFish, the two videos were calibrated by creating a reference volume from four marks with known coordinates recorded against each front of the observation tank facing the camera. The reference volume established a scaled coordinate system from which the three-dimensional spatial coordinates of fish location was derived. Swim paths were reconstructed frame-by-frame from the initial, middle and last five minutes of the observation to provide representative sampling.

Using the Anapaths software (also from Racca Scientific Consulting and JASCO Research Ltd., Virginia, British Columbia, Canada), paths that were closely adjacent to each other and which were consistent with a single swim path were joined together. Paths that were too short (below 2 body lengths) and/or had extremely jagged (unrealistic) swim trajectories were not included in the analysis. The kinematics variables of the swim paths namely, move duration, distance and speed, stop duration, and horizontal and vertical turn angles (i.e. change in direction after a stop in a horizontal and vertical planes) were extracted using the Anapaths software. Every data point from each reconstructed fish swim path was used as an observation value for statistical

analysis. The output files for each replicate tank consisted of a list of recorded - for example - turn angles, which did not differentiate individual fish in the tank.

Analysis of foraging behavior

Twelve equally-spaced one-minute video segments from each replicate tank viewed from one field of view were analyzed for the foraging behavior of herring larvae. Five larvae from each segment were followed visually until they disappeared from the video screen. In total, we have analyzed 1011 herring larvae and 536.1 minutes of video segments for the observation of feeding behavior of 34 and 40-dph larvae. The foraging behavior analyzed were percentage of larvae that performed complete feeding S-strikes and percentage of larvae that initiated but abandoned the feeding S-strike. During prey capture, herring larva bends its body into an aiming S-shaped strike posture upon seeing a prey and then darts forward into the prey to capture it (Munk and Kiørboe 1985, Rosenthal and Hempel 1970, Beyer 1980). A complete feeding strike comprised of the initiation of the S-shaped posture and the subsequent dart action. Whether a strike resulted to a successful capture of prey or not was not taken into account because the magnification used to record the swimming behavior did not provide high confidence to identify the prey item. Checkley (1982) analyzed abandoned feeding in terms of rejected particles where herring larvae undertakes an S-shaped strike posture but not followed by an attack. The same criterion was used in the study. The percentages of complete and abandoned feeding strikes were observed separately, i.e. a larva could have complete or abandoned strikes, both or none at all. Thus, the percentages did not add up to 100%.

Data analysis

Standard lengths were compared among treatments using a Nested-Analysis of Variance with replicates nested in $p\text{CO}_2$ treatments.

The horizontal and vertical turn angle components of the swim paths were analyzed using circular statistics (Batschelet 1981). A non-parametric Mardia-Watson Wheeler test (MWW test) was used to compare distributions of the turn angles between replicates within each treatment, and then between treatments provided that replicates did not show any significant differences. If data showed higher variability between replicates than between treatments, it was evident that treatments had no effect.

For the other variables (stop duration, move duration, move length, and move speed), a non-parametric two-sample Kolmogorov-Smirnov (KS) test was used to

compare the distribution of replicates within treatments and then between treatments, again, provided that replicates did not show any significant differences. When a significant difference was found a Mann-Whitney U-test was applied to examine whether the distribution of one treatment is significantly greater than the distribution of other treatment (Mann and Whitney 1947). A 5% significance level was used in all tests.

Multiple Chi-squared tests were performed to analyze the percentages of larvae with complete feeding S-strikes and abandoned strikes. The variability among replicates within treatment was tested first before treatment comparisons were made.

Results

Growth parameters. Elevated $p\text{CO}_2$ resulted to smaller larvae throughout the duration of the experiment except at 0 and 7-dph (Figure 1). The reduction in standard length increased significantly from 25 to 39-dph.

Swimming behavior. Higher variability within treatments was more frequent in the elevated $p\text{CO}_2$ treatments than in the control group. In the 12 instances of within-treatment variability check, significant differences among replicates were observed six and seven times in medium and high $p\text{CO}_2$ treatments, respectively. In contrast to a single non-homogeneous replicates in the control group (Table 1). Thus, treatment comparisons were not carried out with all treatments due to variability between replicates.

Turn angles. The turn angles were not significantly affected by elevated $p\text{CO}_2$ (Figure 2 HTA, Figure 3 VTA). At 40-dph, horizontal turn angles of the medium treatment (62°) were significantly smaller than the high treatment (67°) (MWW test $p_{\text{M-H}} = 0.023$) but this could not be related with increase in $p\text{CO}_2$ because the control group was not significantly different than medium or high treatments (Figure 2).

Linear variables. Of the linear variables tested, significant difference was only observed in the stop duration (Figure 4), i.e. herring larvae from the medium $p\text{CO}_2$ treatment at 40-dph significantly stopped longer (75th percentile, 0.4s) than larvae in the control group (75th percentile, 0.2s) (KS test: $p < 0.01$; Mann-Whitney U test: $p < < 0.001$). Due to the highly skewed distribution of stop durations, the median values of the control and medium groups were equal.

Elevated $p\text{CO}_2$ did not significantly affect the move durations of herring larvae in both age groups with larvae swimming on average 0.2s (median value) (Figure 5).

KS test of swim speed between control and medium treatments at 40-dph showed a very small significant difference (KS test: $p < 0.05$) (Figure 6). This could be interpreted as a slight decrease in swimming speed of larvae from the medium treatment (median, 14.4 mm s^{-1}) relative to the control (median, 15 mm s^{-1}). However, further testing with Mann-Whitney U test did not show significant difference between control and medium treatments (Mann-Whitney U test: $p = 0.13$).

No treatment comparison was made in move lengths at 34-dph because of high within-treatment variability (Figure 7). While move lengths at 40-dph did not show significant difference between control and medium groups.

Feeding observations. Due to high variability between replicates, treatment comparisons of complete and abandoned feeding strikes were only done at 40-dph and 34-dph, respectively (Figure 8). There was no significant difference in the percentage of larvae with complete feedings strikes between the control and medium treatments at 40-dph (Chi-square test, $p = 0.71$). On the other hand, percentage of herring larvae that aborted their feeding strikes was significantly higher in the high treatment than in the control group at 34-dph (Chi-square test, $p = 0.0001$).

Discussion

The swimming behavior of Atlantic herring larvae is robust to extreme levels of elevated $p\text{CO}_2$. A single significant subtle change was shown from the 12 kinematic variables observed in 34 and 40-dph herring larvae. Herring larvae stopped longer in the medium treatment than those reared under control condition. The subtle change observed here could represent robustness of the swimming performance of herring larvae under more relevant near-future ocean acidification scenario, which is projected to reach $850 \mu\text{atm}$ by the end of the century (IPCC 2007). Similarly, cod larvae observed from the same rearing experiment also only showed subtle behavioral changes under elevated $p\text{CO}_2$ (Maneja et al. 2012). Whether these subtle changes are spurious or have any ecological relevance is unclear.

Although we only observe subtle changes in the swimming behavior of Atlantic herring larvae, it seems that the stop duration is the kinematic variable that is most sensitive to elevated $p\text{CO}_2$. In contrast to reduced stop duration in cod larvae (Maneja et

al. 2012), herring larvae reared in elevated $p\text{CO}_2$ had longer stop durations than those reared in the control environment, specifically a 0.2s or a 100% increase in the 75th percentile of stop durations. For a larva that is slightly negatively buoyant, longer time spent inactive increases their sinking rate. Herring larvae kept in darkness, for example, displayed inactive periods characterized by higher frequency of sinking head first (Batty 1987). The proportion of swimming herring larvae with a length of 21mm can be as high as 93% at an adequate light intensity for feeding (45 lux) and down to 30% in darkness (Batty 1987). Swimming inactivity resulting from increased stop duration for larvae greater than 16mm might translate to greater sinking rate as observed in field surveys in herring larvae in the North Sea (Haslob et al. 2009). However, the time difference in the stop durations was very small and visual observation in the sinking rate was not noticeable. Increased stop duration might have consequences as well in the foraging activity of the larvae since herring larvae continuously search for prey while actively swimming (MacKenzie and Kiørboe 1995).

Herring larvae from the three $p\text{CO}_2$ treatments were equally capable of capturing their prey. Although, the incidence of abandoned feeding strikes was more prevalent in the high $p\text{CO}_2$ treatment herring larvae compared to those in the control. These observations can provide a number of inferences on the feeding behavior of herring larvae. Herring larva's decision to attack or reject a prey is influenced by the type, size, and nutritive value of the particle or prey (Checkley 1982). In our study, herring larvae from all the treatments were given the same prey assemblage and size range. Therefore, differences in prey items could have not influenced the foraging behavior of herring larvae observed here. We could infer that prey-perceptive ability of the herring larvae was somehow affected by elevated $p\text{CO}_2$. In anchovy larvae, failure to complete a feeding sequence is caused by the larva's inability to closely approach the prey while forming the S-shape posture (Hunter 1972). Predatory behavior was shown to be affected by elevated $p\text{CO}_2$, e.g. brown dottyback, a tropical fish species, shifted from preference to avoidance of the smell of injured prey (Cripps et al. 2011). Increase in abandoned feeding strikes could also provide some insights into possible impairment of moto-neural control of prey capture behavior. Performance of highly defined feeding posture with localized bending along the axis of the larva is controlled by specific neural circuits (Budick and O'Malley 2000; Borla et al. 2002). A recent study by Nilsson et al. (2012) showed that elevated $p\text{CO}_2$ hinders the function of a major neurotransmitter receptor, GABA-A, in the vertebrate brain causing olfactory damage in clownfish and loss of

lateralization in damselfish. Although the food concentration given during the behavioral observation was similar in all the treatments, herring larvae from the high $p\text{CO}_2$ treatment may have had sensory overload from the high prey concentration. 25-mm Atlantic salmon larvae experienced perceptual confusion in extremely high prey levels resulting in decrease of prey capture success (Marcotte and Browman 1986; Hauss and Peck 2009).

The current analysis of the effects of elevated $p\text{CO}_2$ on the swimming behavior of Atlantic herring larvae were carried out with significantly different sized larvae. Both the larvae from medium and high treatments were significantly smaller than larvae from the control group, an effect which was also translated into delayed ontogenetic development at day 39-dph (Frommel et al., manuscript in preparation). Despite the decrease in larval size, clear signal of $p\text{CO}_2$ induced behavioral changes were not observed here. In summary, the swimming behavior of Atlantic herring larvae observed at 34 and 40-dph was shown to be robust to extreme $p\text{CO}_2$ levels. The consequences of increased abandoned feeding strikes need a closer investigation.

Acknowledgements

Funding support was provided through the European Marie Curie Initial Training Network "Calcification by Marine Organisms" (CalMarO) and the European Community's Seventh Framework Programme (FP7/2007-2013) "European Project on Ocean Acidification" (EPOCA, grant agreement N211384). The study was also supported by the project "Biological Impacts of Ocean ACIDification" (BIOACID), funded by the German Ministry for Education and Research (BMBF), and by the Norwegian Institute of Marine Research ("Fine-scale behavioral interactions in the plankton" and "Biological effects of ocean acidification" projects to HIB). The experiments were conducted at the Norwegian National Mesocosm Centre, Espegrend, in cooperation with the Department of Biology, University of Bergen and at the Institute of Marine Research's Austevoll Research Station.

Table 1. Summary of statistics. * Significant difference, NS: non-significant difference, NT: no tests were performed because replicates were significantly different within treatments. Non-parametric tests were: Mardia-Watson Wheeler test (MWW test) for turn angles; Kolmogorov-Smirnov test and Mann-Whitney test for linear parameters.

| Swim kinematics | Age (dph) | Treatment | N | Median | Min-max | Statistical significance: between replicates | Statistical significance: between treatments |
|-----------------|-----------|-----------|------|--------|----------|--|--|
| VTA | 34 | C | 1813 | 19° | 0-133° | NS | NT |
| | | M | 1350 | 20° | 0-143° | * | |
| | | H | 1197 | 17° | 0-105° | * | |
| | 40 | C | 1125 | 22° | 0-152° | NS | C=M |
| | | M | 1227 | 21° | 0-156° | NS | |
| | | H | 1660 | 19° | 0-140° | * | |
| HTA | 34 | C | 1757 | 71° | 0-180° | NS | C=H |
| | | M | 1294 | 68° | 0-180° | * | |
| | | H | 1160 | 69° | 0-180° | NS | |
| | 40 | C | 1072 | 65° | 0-179° | NS | M≠H C=M C=H |
| | | M | 1169 | 62° | 0-180° | NS | |
| | | H | 1603 | 67° | 0-180° | NS | |
| SD | 34 | C | 2009 | 0.1 | 0.1-3.8 | NS | C=H |
| | | M | 1475 | 0.1 | 0.1-2.3 | * | |
| | | H | 1311 | 0.1 | 0.1-1.6 | NS | |
| | 40 | C | 1248 | 0.1 | 0.1-2.2 | NS | C≠M |
| | | M | 1342 | 0.1 | 0.1-3.4 | NS | |
| | | H | 1858 | 0.1 | 0.1-2.8 | * | |
| MD | 34 | C | 2385 | 0.2 | 0.1-2.8 | NS | C=H |
| | | M | 1737 | 0.2 | 0.1-1.9 | * | |
| | | H | 1532 | 0.2 | 0.1-1.7 | NS | |
| | 40 | C | 1505 | 0.2 | 0.1-2.3 | NS | C=M |
| | | M | 1585 | 0.2 | 0.1-2.5 | NS | |
| | | H | 2143 | 0.2 | 0.1-2.4 | * | |
| MS | 34 | C | 2293 | 16.1 | 7.5-107 | NS | C=H |
| | | M | 1679 | 14.7 | 7.5-86.3 | * | |
| | | H | 1482 | 15.9 | 7.5-90 | NS | |
| | 40 | C | 1463 | 15.0 | 7.5-90.9 | NS | C≠M |
| | | M | 1541 | 14.4 | 7.5-78.7 | NS | |
| | | H | 2060 | 14.1 | 7.5-89.5 | * | |
| ML | 34 | C | 2385 | 4.7 | 0.9-59.2 | * | NT |
| | | M | 1737 | 3.9 | 0.9-52.3 | * | |
| | | H | 1532 | 4.6 | 0.9-55.4 | * | |
| | 40 | C | 1505 | 4.6 | 0.9-47.8 | NS | C=M |
| | | M | 1585 | 4.0 | 0.9-48.9 | NS | |
| | | H | 2143 | 3.5 | 0.9-44.6 | * | |

Figure 1. Mean standard length of Atlantic herring larvae sampled at various periods during the mesocosm experiment in Espeyrend Marine Station from March-May 2010. Whiskers denote 95% confidence interval. Recording of the swimming behavior was done at 34 and 40-dph.

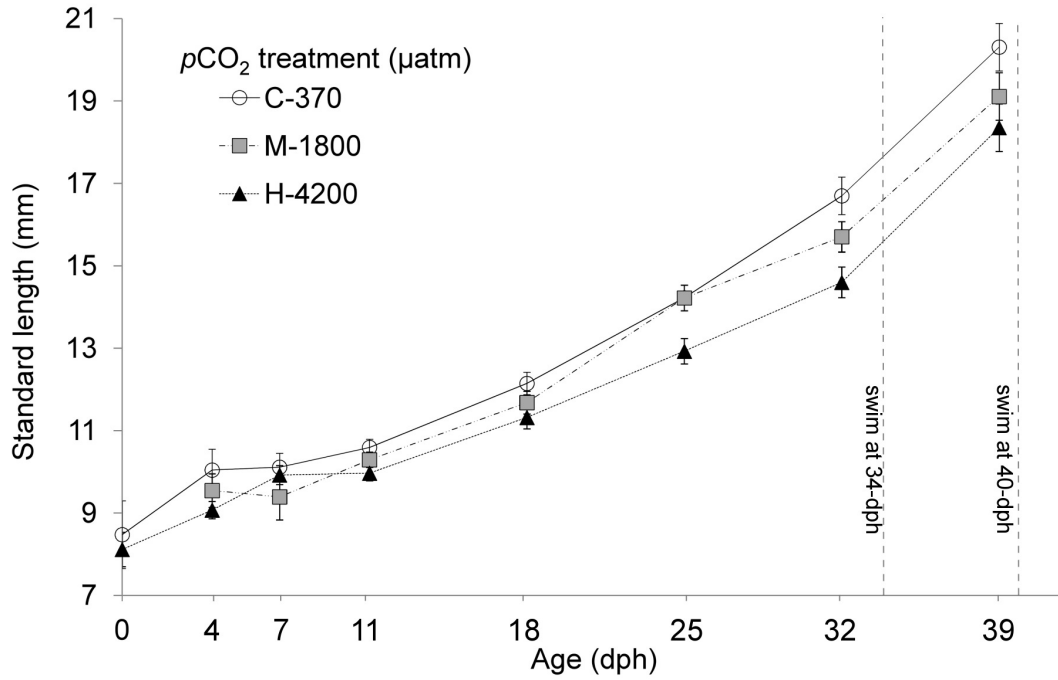


Figure 2. Horizontal turn angles of (a) 34-dph and (b) 40-dph Atlantic herring larvae (*Clupea harengus* L.) from three $p\text{CO}_2$ levels (C-380 μatm , M-1800 μatm , H-4200 μatm). Lines from the center denote mean vector angle and arcs at the end of the lines represent 95% confidence interval for each $p\text{CO}_2$ treatment.

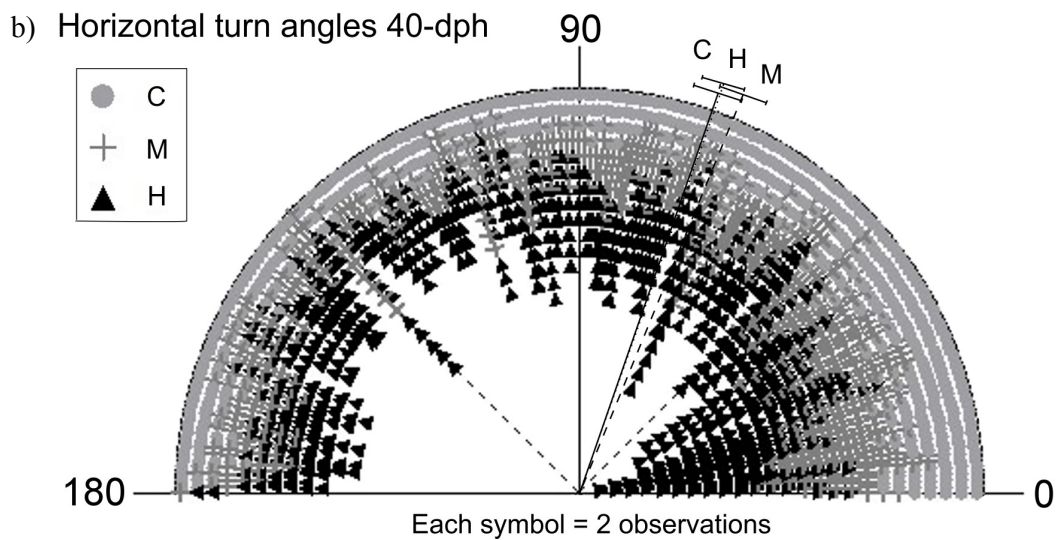
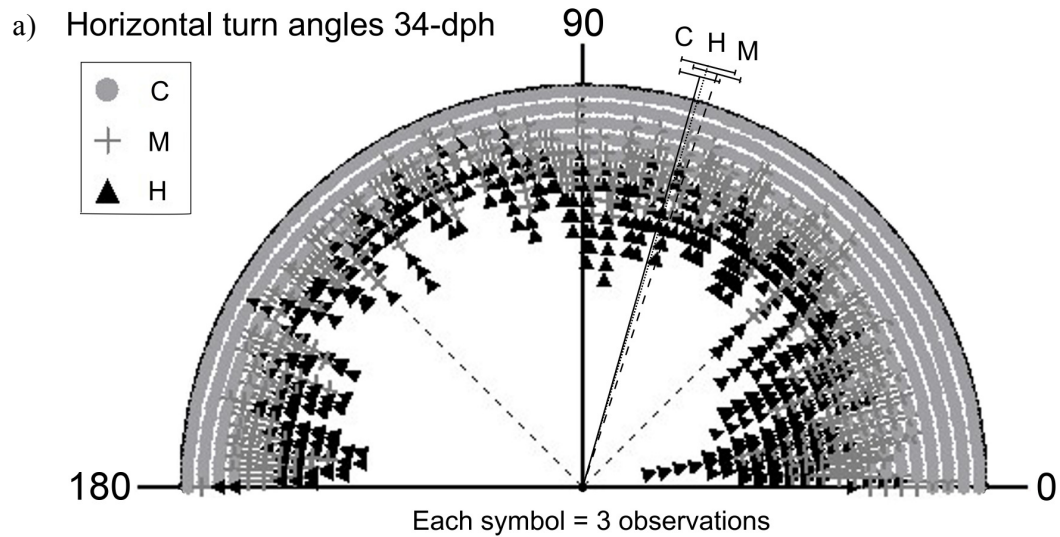


Figure 3. Vertical turn angles of (a) 34-dph and (b) 40-dph Atlantic herring larvae (*Clupea harengus* L.) from three $p\text{CO}_2$ levels (C-380 μatm , M-1800 μatm , H-4200 μatm). Lines from the center denote mean vector angle and arcs at the end of the lines represent 95% confidence interval for each $p\text{CO}_2$ treatment.

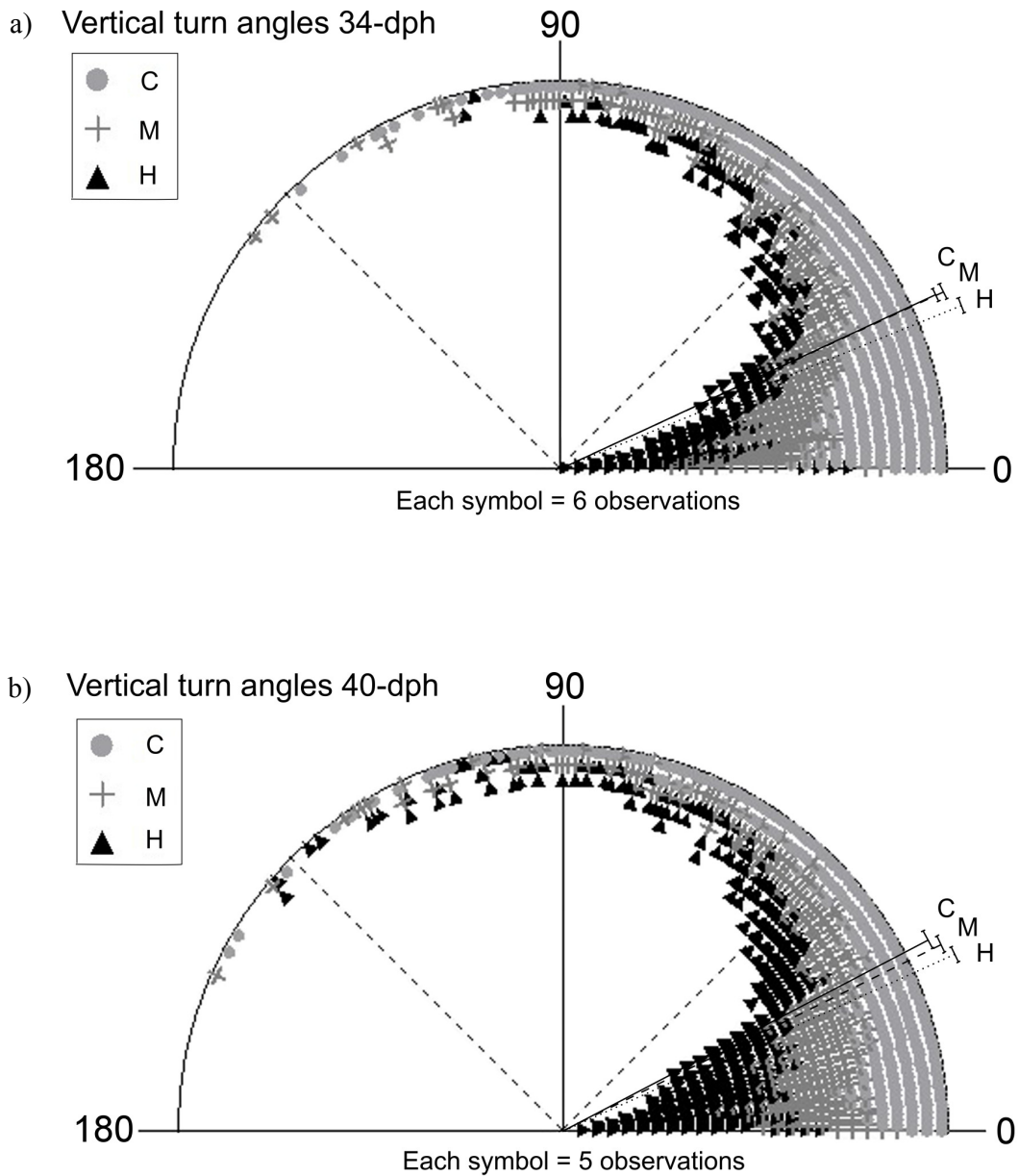


Figure 4. Cumulative fraction plots for the Kolmogorov-Smirnov tests of stop duration of Atlantic herring larvae (*Clupea harengus* L.) at (a) 34-dph and (b) 40-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Results of comparisons among $p\text{CO}_2$ treatments are indicated by D_{max} and p -value.

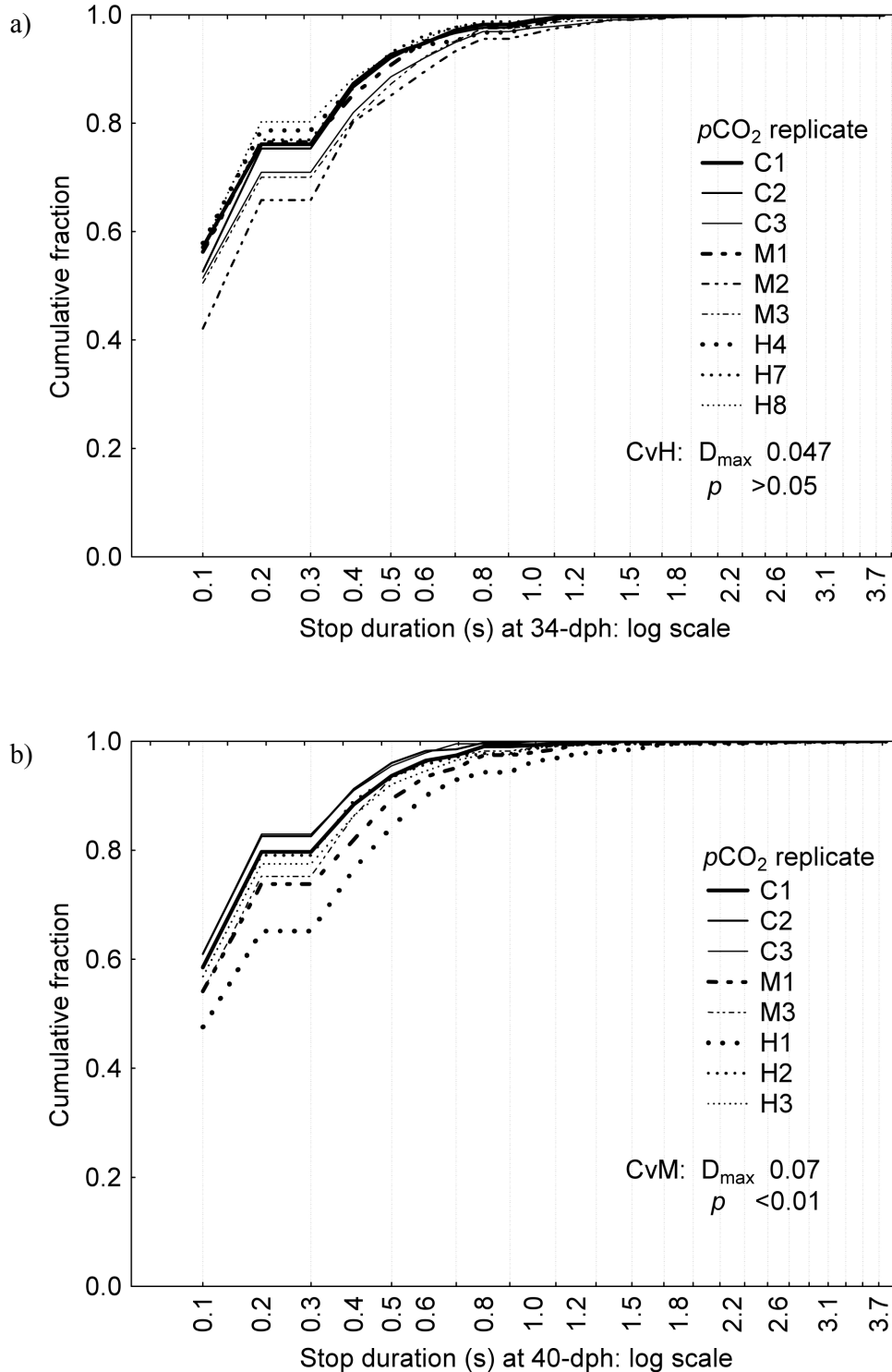


Figure 5. Cumulative fraction plots for the Kolmogorov-Smirnov tests of move duration of Atlantic herring larvae (*Clupea harengus* L.) at (a) 34 and (b) 40-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Results of comparisons among $p\text{CO}_2$ treatments are indicated by D_{max} and p -value.

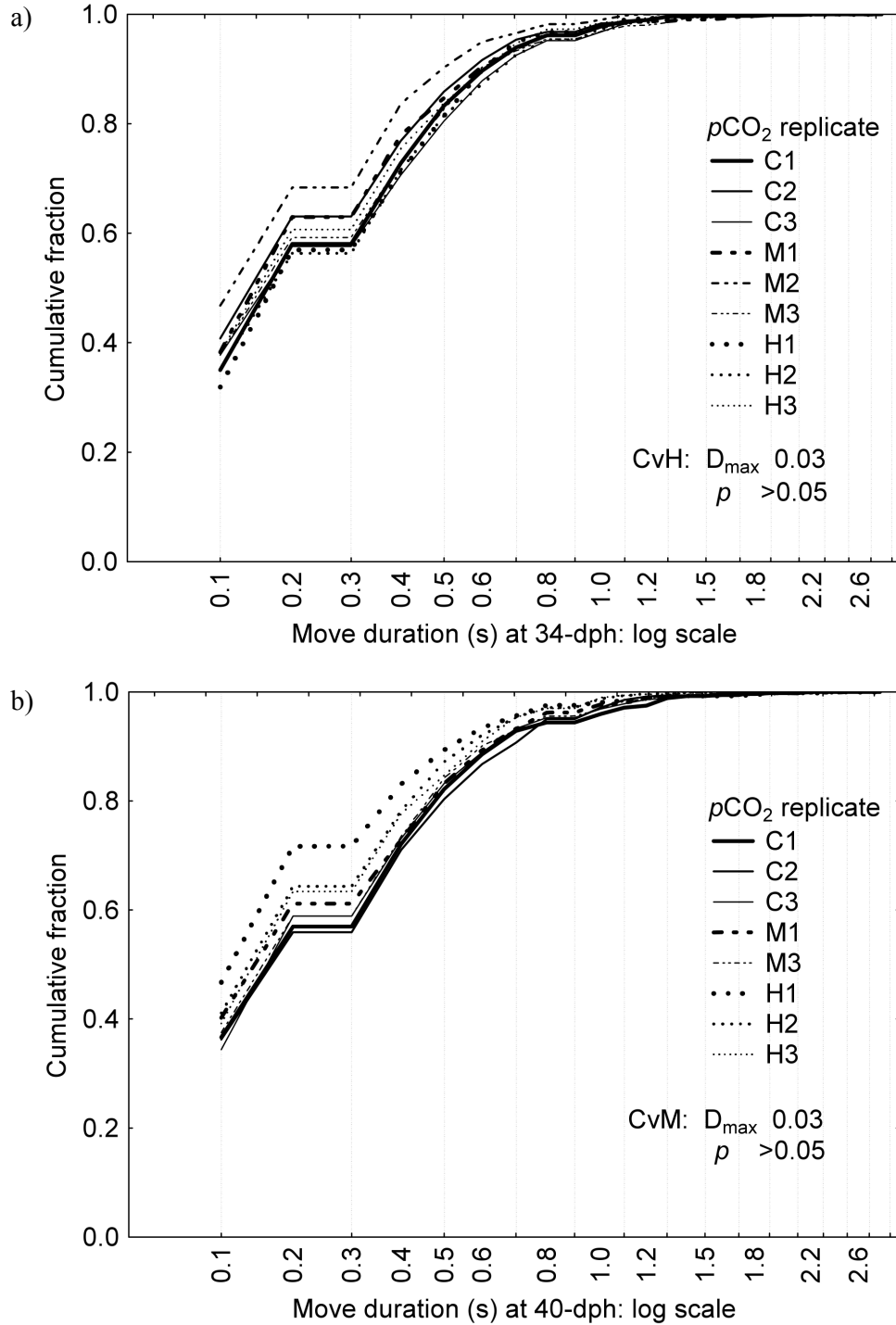


Figure 6. Cumulative fraction plots for the Kolmogorov-Smirnov tests of swimming speed of Atlantic herring larvae (*Clupea harengus* L.) at (a) 34 and (b) 40-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Results of comparisons among $p\text{CO}_2$ treatments are indicated by D_{max} and p -value.

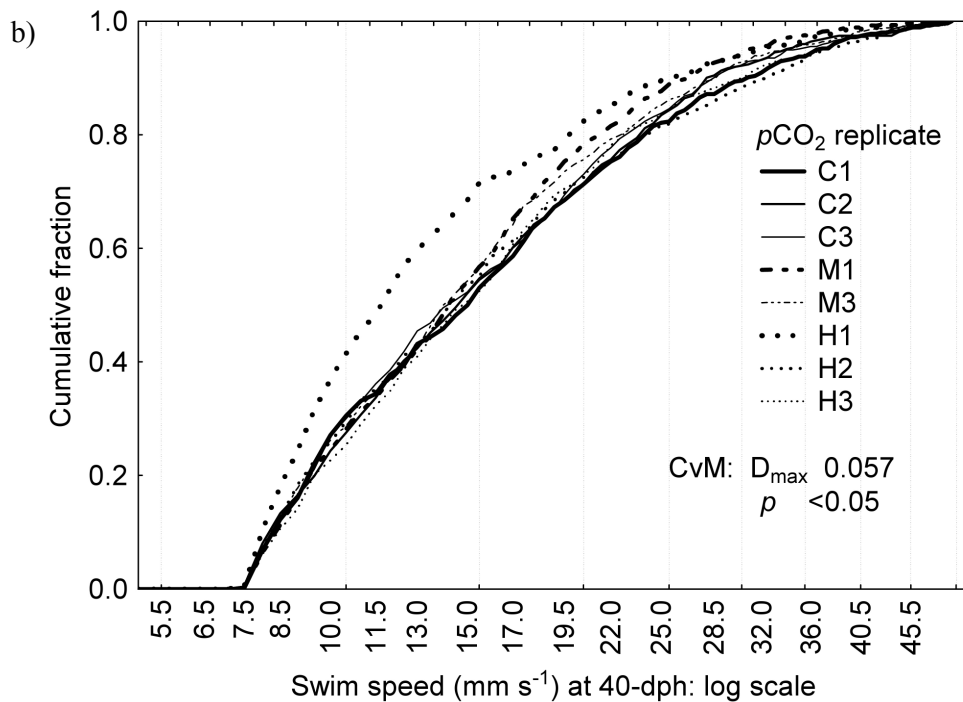
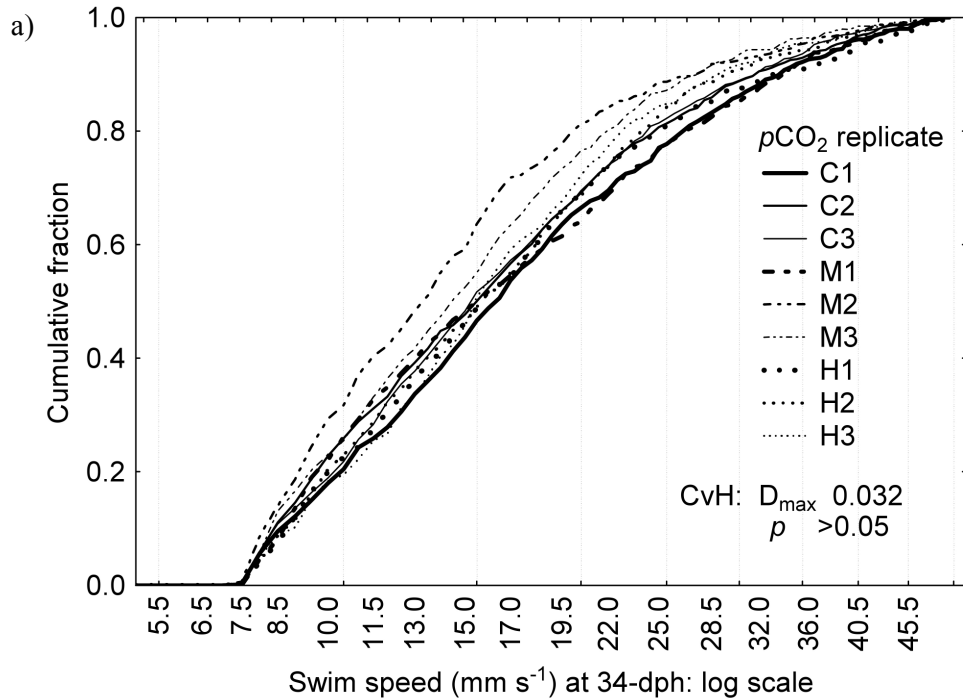


Figure 7. Cumulative fraction plots for the Kolmogorov-Smirnov tests of swimming distance of Atlantic herring larvae (*Clupea harengus* L.) at (a) 34 and (b) 40-dph under three $p\text{CO}_2$ levels (C=370 μatm , M=1800 μatm , and H=4200 μatm). Results of comparisons among $p\text{CO}_2$ treatments are indicated by D_{max} and p -value.

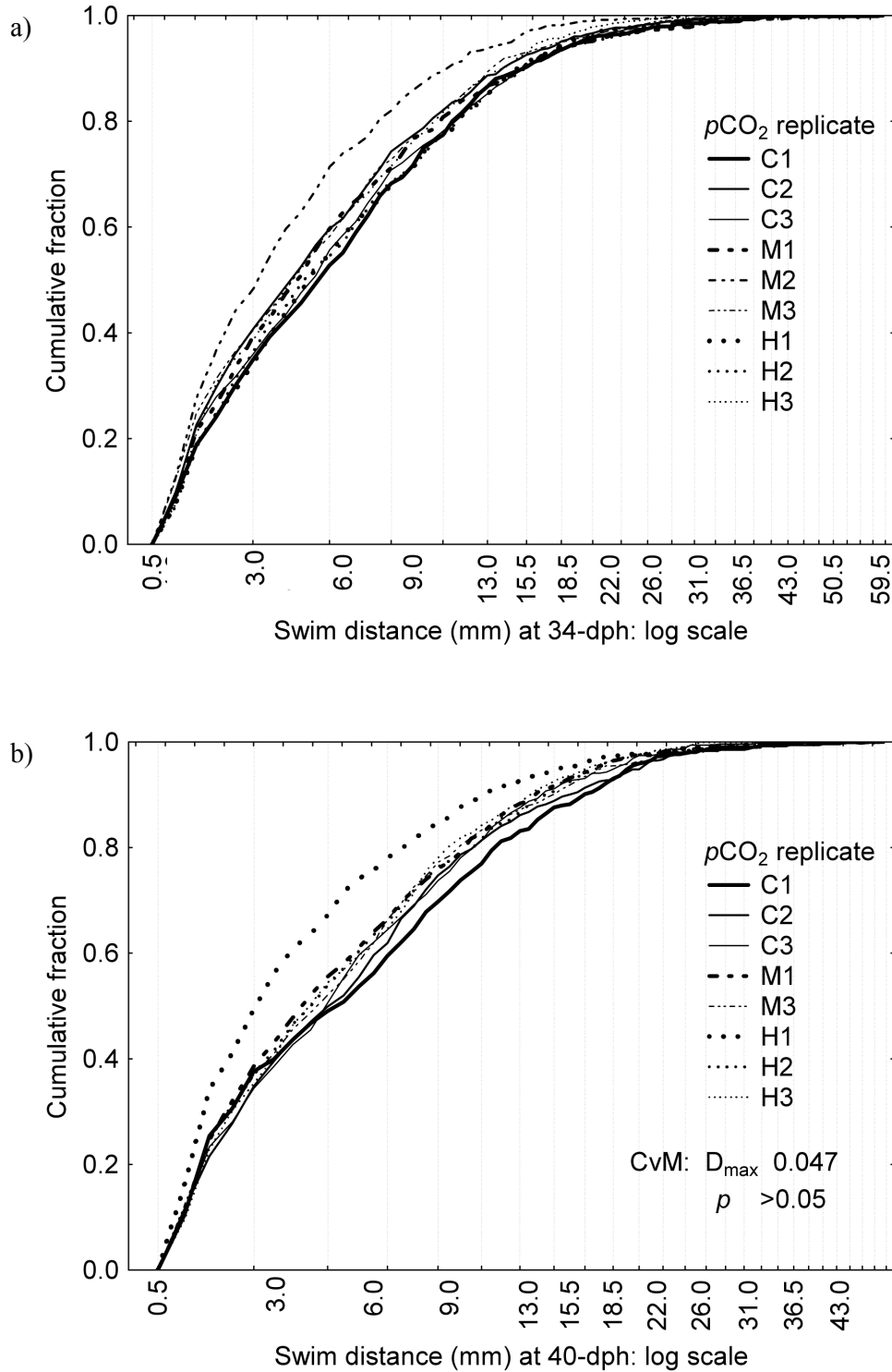
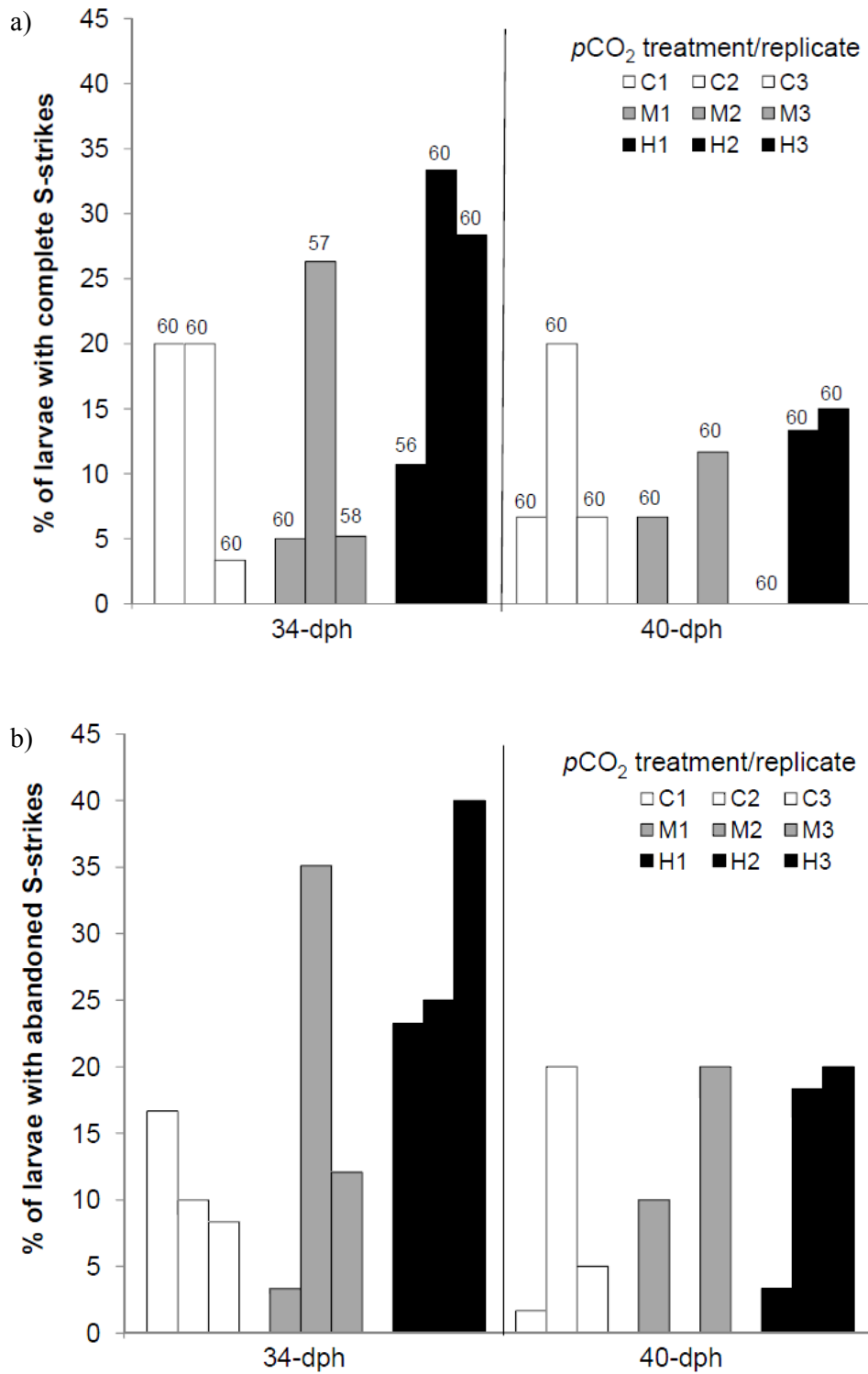


Figure 8. Percentage of observed Atlantic herring larvae with a) complete and b) abandoned feeding S-strikes. Number of larvae observed per replicate are provided above each bar plot.



Additional preliminary data on Atlantic herring otoliths

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Description: Both the sagittae and lapilli were extracted from Atlantic herring larvae co-reared with the cod larvae under three $p\text{CO}_2$ treatments in the Espesrend mesocosm experiment. The same statistical analyses described in Chapter I were conducted to analyze the effects of $p\text{CO}_2$ treatments on the differences in otolith area and fluctuating asymmetry. The otoliths analyzed were from 25, 32, and 39-dph herring larvae.

Analysis:

1. The growth of herring otoliths was significantly reduced at elevated $p\text{CO}_2$ concentrations both in the lapillus and sagitta particularly at 32 and 39-dph (Figure 1). The differences in growth was more pronounced in the sagitta perhaps due to the larger increase in sizes of the sagitta with fish size.
2. The magnitude of fluctuating asymmetry did not vary significantly among $p\text{CO}_2$ treatments (Figure 2).

Implications: The decrease in otolith growth in Atlantic herring larvae in response to increase in $p\text{CO}_2$ concentrations was in contrast to the response of the otoliths of cod larvae. The differences could provide an insight into the differences in the efficiency of acid-base regulation between the two species and the associated metabolic costs in maintaining relatively high intra- and extracellular pH. Despite the decrease in larval and otolith growth, the magnitude of fluctuating asymmetry did not increase with $p\text{CO}_2$ concentration. This could indicate similar carbonate chemistry conditions between the left and right endolymphs.

Figure 1. Mean surface area of a) lapillus and b) sagitta (both normalized to fish standard length, SL) from Atlantic herring larvae (*Clupea harengus* L.) grown at three $p\text{CO}_2$ treatments (C=370 μatm , M=1800 μatm , H=4200 μatm). Whiskers denote 95% confidence interval. Different letters above bars denote significant differences between $p\text{CO}_2$ treatments based on Analysis of Covariance test with SL as the covariate (at 5% significance level). Numbers inside bars indicate sample sizes.

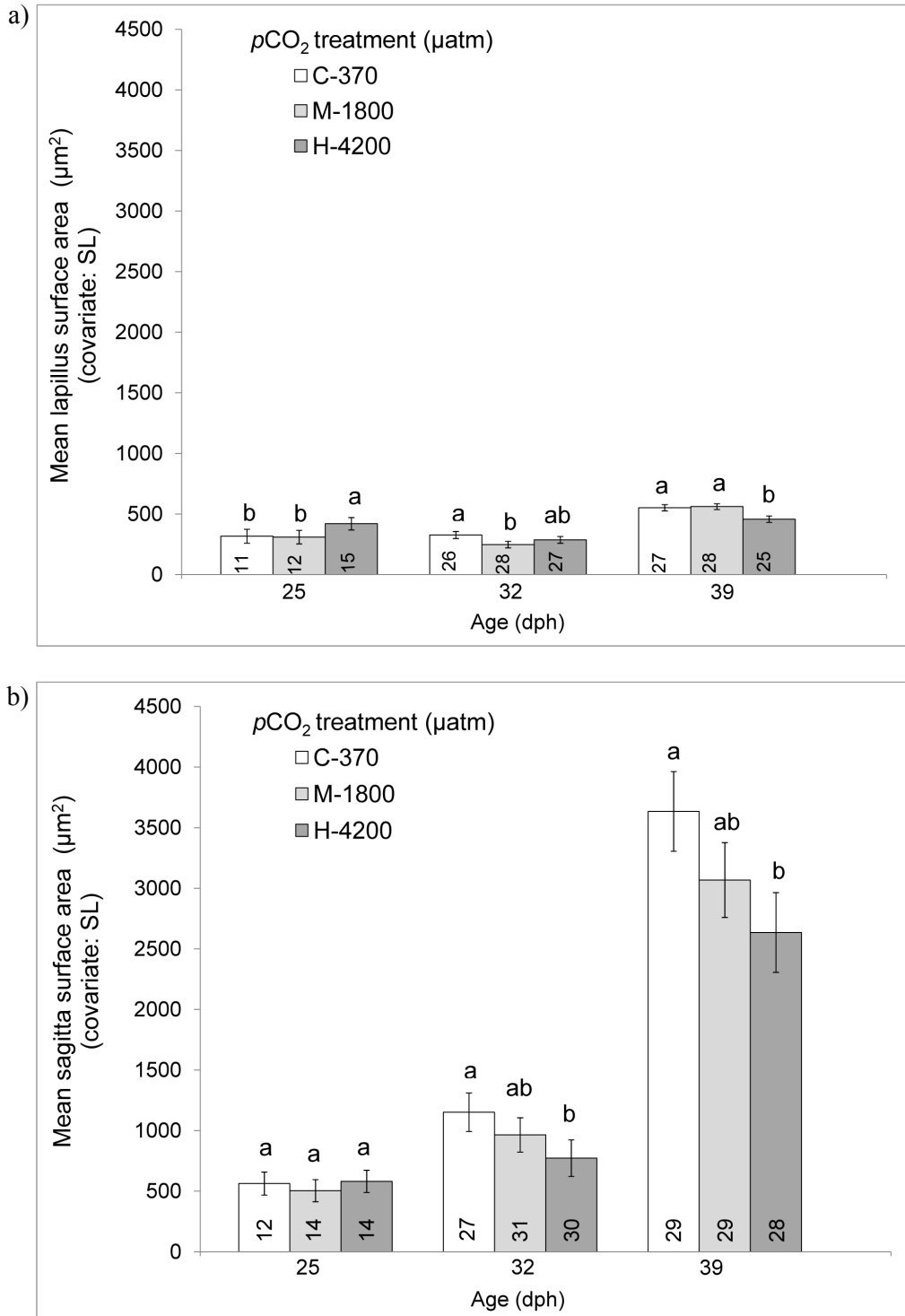
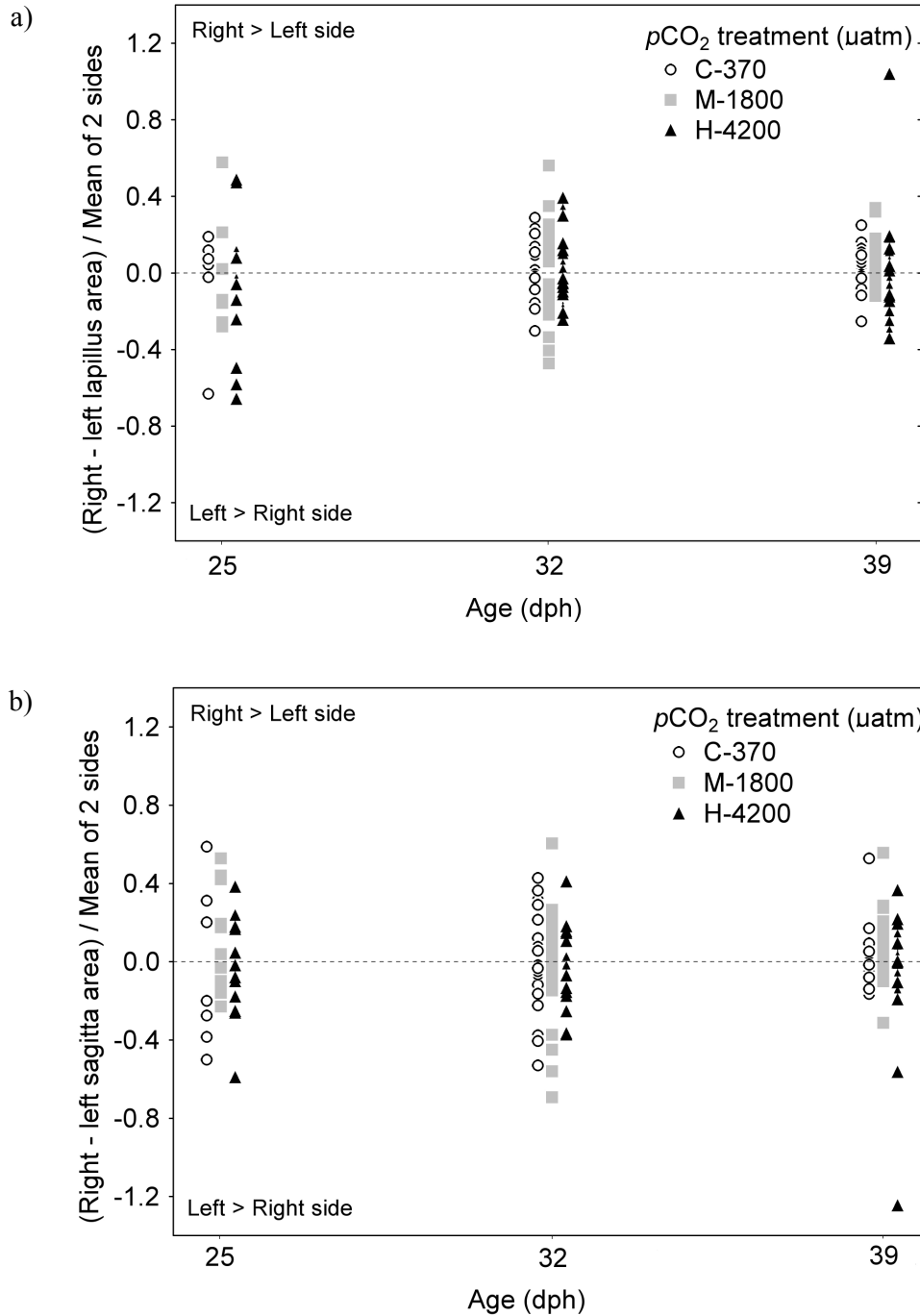


Figure 2. Scatterplots of the signed difference of otolith surface area between the right and left otoliths of Atlantic herring larvae (*Clupea harengus* L.) grown under three $p\text{CO}_2$ concentrations (Control=370 μatm , Medium=1800 μatm , High=4200 μatm). Signed difference was normalized by dividing with the mean surface area of the two sides. Offset in the x-axis was used to display the data per $p\text{CO}_2$ treatment. a) Lapillus b) Sagitta.



Chapter IV

Chapter IV

Severe tissue damage in Atlantic cod larvae under increasing ocean acidification

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Ocean acidification (OA), caused by increasing atmospheric concentrations of CO₂ (Caldeira and Wickett 2003; IPCC 2007; Caldeira and Wickett 2005), is one of the most critical anthropogenic threats to marine life. Changes in seawater carbonate chemistry have the potential to disturb calcification, acid-base regulation, blood circulation and respiration, as well as the nervous system of marine organisms, leading to long-term effects such as reduced growth rates and reproduction (Fabry et al. 2008; Pörtner et al. 2004). In teleost fishes, early life-history stages are particularly vulnerable as they lack specialized internal pH regulatory mechanisms (Morris et al. 1989; Sayer et al. 1993). So far, impacts of relevant CO₂ concentrations on larval fish have been found in behavior (Sayer et al. 1993; Munday et al. 2009b) and otolith size (Munday et al. 2009b; Munday et al. 2010; Munday et al. 2011; Checkley et al. 2009), mainly in tropical, non-commercial species. Here we show detrimental effects of OA on the development of a mass-spawning fish species of high commercial importance. We reared Atlantic cod larvae at three levels of CO₂ (1) today, (2) end of next century, and (3) an extreme, coastal upwelling scenario, in a long-term (2½ months) mesocosm experiment. Exposure to CO₂ resulted in severe to lethal tissue damage in many internal organs, with

the degree of damage increasing with CO₂ concentration. As larval survival is the bottleneck to recruitment, OA has the potential to act as an additional source of natural mortality, affecting populations of already exploited fish stocks.

Present average CO₂ levels in the atmosphere have already exceeded 380 ppmv and are predicted to further increase by 0.5% per year throughout this century, a rate 100 times faster than seen in the past 650,000 years (Siegenthaler et al. 2005). Approximately a third of excess CO₂ in the atmosphere will be dissolved in ocean waters, leading to an estimated drop in pH of 0.4 units ($p\text{CO}_2 \sim 1000 \mu\text{atm}$) globally by the year 2100 and up to 0.8 units ($p\text{CO}_2 \sim 2000 \mu\text{atm}$) by the year 2300 (Caldeira and Wickett 2003; IPCC 2007; Caldeira and Wickett 2005). Locally, the effects can be even more severe, especially in coastal regions with upwelling of oxygen poor, CO₂ rich water, and $p\text{CO}_2$ values above 4000 μatm in the future could be reached in habitats where cod larvae occur (Thomsen et al. 2010).

The Atlantic cod, *Gadus morhua*, has a wide distribution throughout the North Atlantic. Several eastern Atlantic populations are found from the high Arctic down to North and Baltic Sea, where they experience very different conditions in terms of temperature, salinity, oxygen and present $p\text{CO}_2$ levels. For example, in the Baltic Sea $p\text{CO}_2$ concentrations up to 2300 μatm have recently been measured in the Kiel Fjord (Thomsen et al. 2010) close to where the eastern Baltic cod stock spawns, and 1200 μatm in the deep waters of the Bornholm Basin (Frommel et al. 2012b), an important spawning ground for the western Baltic cod stock. The Norwegian coastal cod used in this study live and spawn in a large number of fjords along the entire Norwegian coast and near the Lofoten Islands (Nordeide 1998). These high latitudes are assumed to be particularly impacted by future ocean acidification, due to cold water, high primary productivity and melting of sea ice (Fabry et al. 2009; Steinacher et al. 2009; Bellerby et al. 2005), and pH values are predicted to approach 7.7 over most of the coastal Arctic Ocean by the year 2100 (Denman et al. 2011). Recent models even calculate that the decrease in pH could be doubled in some parts of the Arctic Ocean as a result of gas hydrates destabilized by warming ocean temperatures releasing large amounts of methane which in turn are respired by methanogenic bacteria to CO₂ (Biastoch et al. 2011).

Adult teleost fishes are thought to be relatively robust to changes in ambient pH as they are able to control their acid-base balance by bicarbonate buffering, mainly across the gills and via the kidney (Gilmour and Perry 2009; Perry and Gilmour 2006).

However, early life-history stages lacking gills may not be as competent in regulating their internal acid-base balance (Morris et al. 1989; Sayer et al. 1993) and are thus predicted to be impacted more heavily by increasing $p\text{CO}_2$ levels.

In this study, we experimentally tested this prediction and exposed larvae of Norwegian coastal cod to three levels of $p\text{CO}_2$ (control: 380 μatm , medium: 1800 μatm and high: 4200 μatm) from newly fertilized eggs to seven weeks post-hatch (for full methods, see supplementary material). Cod larvae are difficult to rear in the laboratory and require space, near-natural conditions and live prey in order to survive. Our large (2300 L) outdoor mesocosms mimicked natural conditions for the larvae as closely as possible, including flow-through of fresh water and natural zooplankton prey from the fjord. Using such large experimental units only allowed three replicates. It also limited the ability to monitor larval numbers inside the tanks, and since dead larvae decayed before they could be counted, our direct mortality estimates are restricted to the differences in the number of larvae placed into the tanks at the beginning (10 000 per tank) and the ones counted out at the end of the experiment (control: 153 ± 134 , medium: 324 ± 513 , high: 73 ± 70 ; mean \pm S.D.), which revealed no significant difference. However, a retrospective power analysis informed us that we might have missed a mortality difference as large as 50% (setting $\alpha=5\%$) due to the small replicate number ($n=3$) and substantial variation within treatments.

Larval growth was positively affected by high $p\text{CO}_2$ between 25 and 46 days post hatch (dph) (Figure 1). At 32 dph cod larvae from the high treatment had attained 59 % more dry weight relative to the control while the medium treatment was not significantly different (for statistics see Supplementary Table 1). Interestingly, growth in the control animals stagnated between 25 and 32 dph indicating that the larvae were re-allocating their energy from growth to development of internal organs. This age coincides with the developmental stages 8 and 9, a phase of intense transition where critical structural changes take place in all major landmarks (Hunt von Herbing et al. 1996b; Morrison 1987). Most importantly, the respiratory, feeding and locomotion structures greatly increase their function. As larvae grow in size, they become limited by cutaneous respiration as their body volume to surface ratio decreases and they must switch from cutaneous to branchial respiration. At stage 9 in cod larvae, respiration becomes fully branchial as gill filaments increase in number and secondary lamellae are formed, while the gills become completely covered by the opercular membrane, augmenting unidirectional flow over the gills. The larvae under increased $p\text{CO}_2$, however, continued

to allocate energy to growth, at the cost of organ development, and may have outgrown the critical surface to volume ratio necessary for effective cutaneous acid-base regulation. Furthermore, the observed increase in growth was based on lipid instead of protein biosynthesis. One effect when larvae cannot extrude protons is respiratory acidosis, which interferes with different metabolic pathways (Heisler 1989) and may cause a shift from aerobic to anaerobic metabolism (Michaelidis et al. 2007). This in turn can influence protein biosynthesis (Langenbuch and Pörtner 2003). While the protein content and RNA/DNA ratio – as indicator for the protein synthesis capacity - remained relatively constant, the amount of lipid storage peaked during the same time interval as the growth in the treatment larvae (Figure 2). At 32 dph cod larvae from the medium treatment had 33%, and from the high treatment 43% more lipid content than in the control (Supplementary Table 1). No significant difference in fatty acid composition could be identified (ANOVA, $p > 0.05$). Higher lipid content, although an energy store, may not necessarily be beneficial to the organism when it accumulates as droplets in specific organs.

Our determination of the critical phase of organ developments and internal re-adjustments coincided with major histological damage observed in the larvae under elevated $p\text{CO}_2$ treatments. Severe tissue damage was found in the liver, pancreas, kidney, eye and the gut of larvae 32 dph (Figure 3), with the degree of damage significantly increasing with $p\text{CO}_2$ concentration (Figure 4, for statistics see Supplementary Table 2). Throughout the livers of CO_2 treated larvae, large lipid vacuoles were observed. While typical lipid vacuoles of control animals were in the order of 3-4 μm in diameter, impacted cod had lipid vacuoles in the order of 7-9 μm in diameter. Further, atypical liver morphology and necrotic hepatocytes were found in the medium and high treatments, respectively. Enlarged lipid vacuoles in the liver result from lysosomal dysfunction and the breakdown of the lysosomal vascular system in the hepatocytes, a response often found in fish from chemical pollution (Köhler 1991), which can lead to the observed necrosis. Liver damage as a consequence of high $p\text{CO}_2$ concentrations has previously been found in freshwater fish (Good et al. 2010), as well as in isolated Antarctic fish hepatocytes (Langenbuch and Pörtner 2003). Similar to the liver, there was evidence of vacuolation in the epithelial cell cytoplasm of the kidney, with changes in the staining characteristics. Further, loss in structural integrity of the pronephric tubules and atrophy was observed. Atrophy may be a reflection of cellular dysfunction or, possibly, a breakdown in the desmosomes that bind adjacent cells together. In the high

CO₂ treatment, some of the neck cells of kidney tubules stained darker and showed signs of breaking up, an indication of organ failure. In the pancreas, the damage consisted mainly of alterations to tissue architecture whereby the normal pyramidal exocrine cells sitting on a well defined basement membrane were replaced by rounded cells on an irregular basement membrane and the rosettes of the acini that form around zymogen granules were absent. Damage to the eyes was mainly visible as vacuoles associated with the choroid layer and between the pigmented layer and the outer layer of the cones. Additionally, the pigmented layer often had an irregular profile at increased $p\text{CO}_2$ concentrations. In the gut, the connective tissue was found to be highly fragile with the gut epithelium readily detaching from the basement membrane. Additionally, bacteria were present in the gut lumen and in the connective tissues lying between the basement membrane and the gut epithelium in many of the samples from 32 dph. Bacterial infection and high parasite load may be an indication of a weakened immune system. There were indications of a possible impact on the structure of the musculature in the high treatment but as this parameter is highly dependent on the plane of section, this parameter was considered unsafe. No effects were found in the heart, gills, skeleton or skin.

Most of the histological damages found were classified as regressive changes that terminate in functional impairment or loss of the organ and involve deposits, architectural and structural alterations, degeneration, atrophy and necrosis (after Bernet et al. 1999). The health status of each larvae examined, calculated by the total index *Tot-I*, revealed that 12% of the larvae in the medium treatment and 75% of the larvae in the high $p\text{CO}_2$ treatment had severe damage in multiple tissues (Figure 5). After the onset of gill mediated acid-base regulation, these damages disappeared and no effect of CO₂ was found at day 46 (for statistics see Supplementary Table 3).

This study is the first to demonstrate widespread tissue damage as a result of OA during a critical life-cycle phase within a mass-spawning, commercially important fish. Our data complement other studies that found behavioral effects in response to elevated $p\text{CO}_2$ in tropical fish that have a very different developmental pattern and a much faster developmental rate (Munday et al. 2009b; Munday et al. 2010; Munday et al. 2009c). Laboratory experiments are always limited as they simulate increasing CO₂ at a rate much higher than predicted and therefore neglect the potential for genetic adaptation. However, as cod are long-lived fish with a relatively long generation time, the pace of

evolutionary adaptation to cope with OA is probably relatively slow on absolute time scales. Furthermore, like many other commercially exploited fish, cod already experience high selection pressure via fisheries, in addition to other environmental stressors such as pollution, temperature, salinity and oxygen changes. Although we did not directly test for mortality rates, our data on severe tissue damage suggest that OA will negatively impact the recruitment of mass spawning fishes via enhanced mortality rates. Adverse effects of OA have thus to be added to the growing list of anthropogenic disturbances affecting already exploited fish stocks.

Acknowledgements: Funding was provided through the European Community's Seventh Framework Programme (FP7/2007-2013) "European Project on Ocean Acidification" (EPOCA, grant agreement N211384), the European Marie Curie Initial Training Network "Calcification of Marine Organisms" (CalMarO) and the project by German Ministry for Education and Research (BMBF) "Biological Impacts of Ocean ACIDification" (BIOACID). The experiments were conducted at the Norwegian National Mesocosm Centre, Espesund, in cooperation with the University of Bergen. The authors are grateful to Prof. Richard Bellerby and his lab for assistance with the carbonate chemistry and to Haakon Otteraa, Vibeke Lokøy, Frank Midtøy and Dr. Chris Eizaguirre for various support.

Author contributions:

A.Y.F., R.M., A.J.G, A.F., U.P. and C.C. designed the experiment;

A.Y.F., R.M., A.J.G, A.F. and C.C. performed the experiment;

D.L. performed the histological analysis and wrote the section on that topic;

A.M.M. performed the lipid analysis;

A.Y.F, C.C. and T.R. analyzed data;

A.Y.F. and T.R. wrote the main paper;

All authors discussed the results and implications and commented on the manuscript at all stages.

Figure 1. Larval growth in dry weight over the entire experimental period. Mean ln dry weight (DW) with standard deviation for each of the three treatments over 7 weeks post hatch. Asterisks indicate significant differences between control and high treatment. N= 60 larvae per replicate. For statistics see Supplementary Table 1.

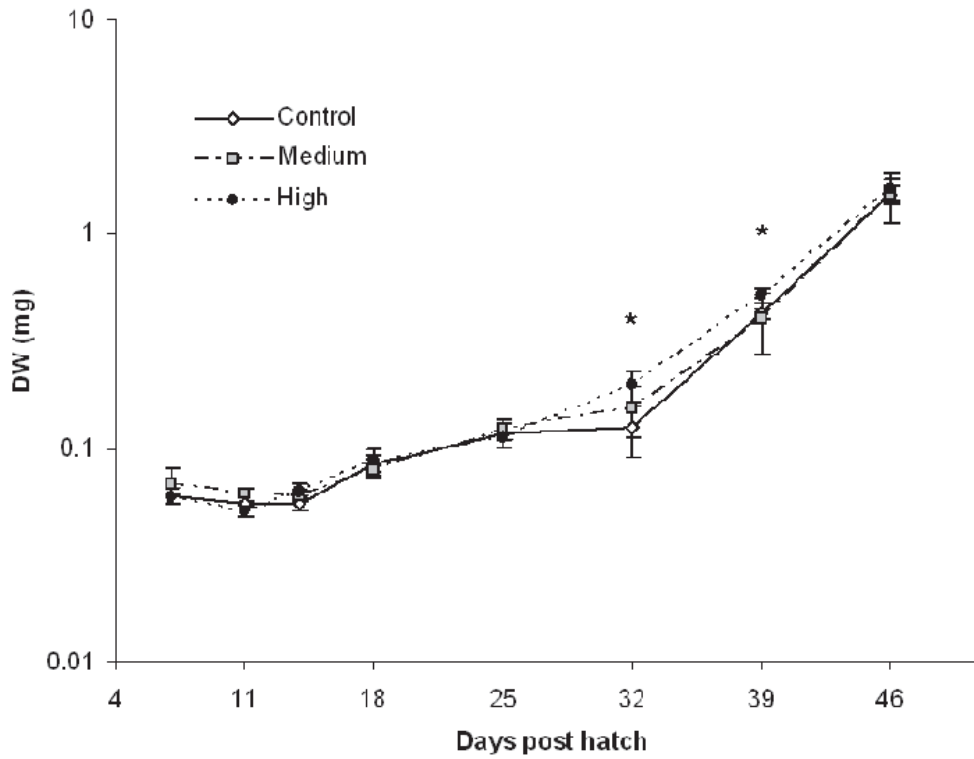


Figure 2. Lipid content of larval cod for the last three sampling intervals.

Mean lipid content of three replicates as percent of dry weight with standard deviation for each of the three treatments (control = white bars, medium = grey bars, high = black bars) at 32 dph, 39 dph and 46 dph. Letters indicate significant differences (for test statistics, see Supplementary Table 1). N= 30 larvae per replicate.

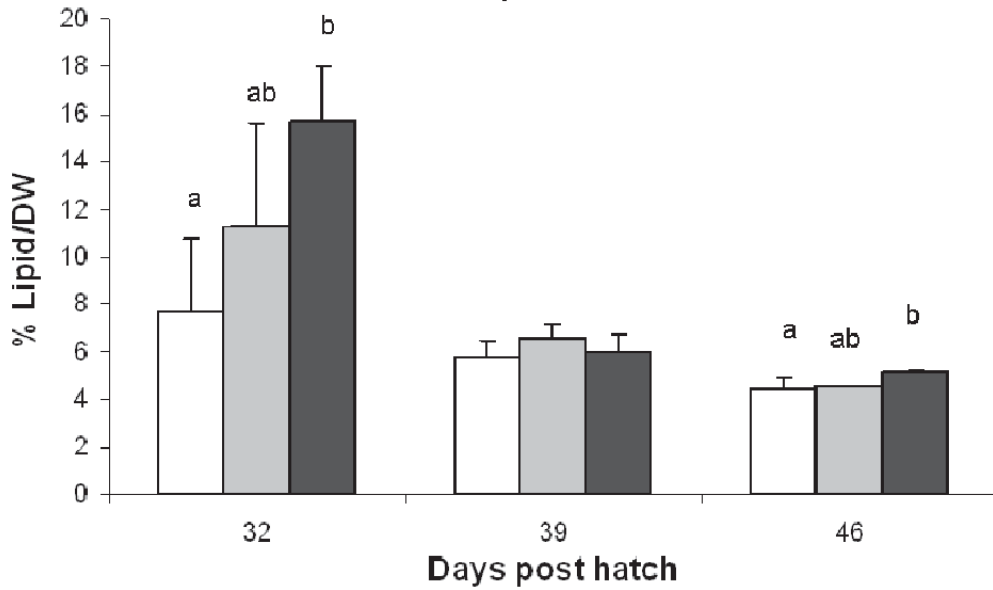


Figure 3. Tissue damage from histological sections in larvae under increased $p\text{CO}_2$. Histological sections of different organs in cod larvae at three different $p\text{CO}_2$ treatment levels (control, medium and high). Structures seen: Liver: enlarged lipid vacuoles (lv), necrotic hepatocytes (nec); Kidney: pronephric tubules (t), neck cells breaking up (nc), vacuoles (v), atrophy (a); Pancreas: rosettes of acini (ra), rounded cells (rc), basement membrane (bm); Eye: pigmented layer (pi), vacuoles (v); Gut: gut epithelium (ge), basement membrane (bm), bacteria (bac).

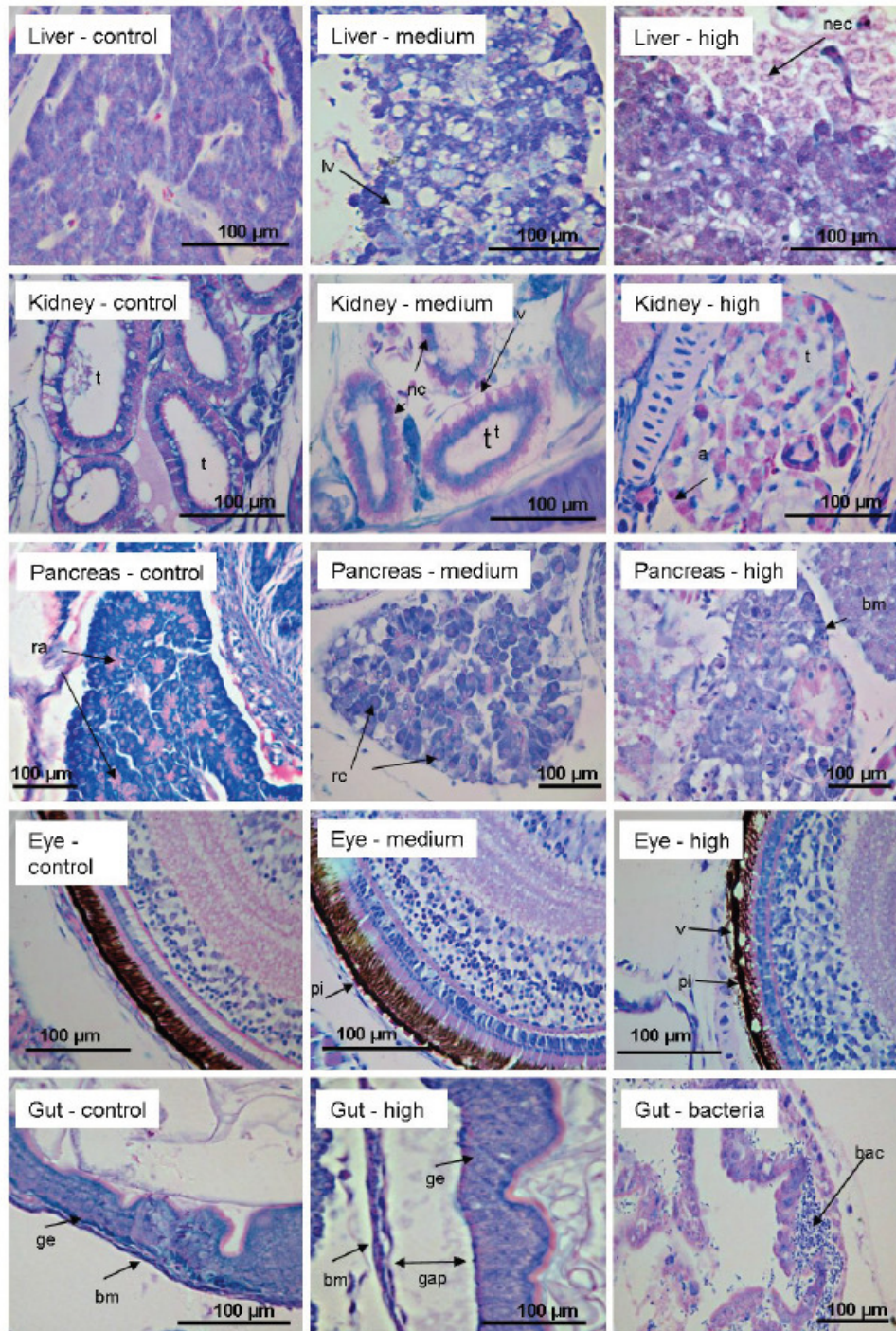


Figure 4: Quantification of degree of damage in various organs at increasing $p\text{CO}_2$. Mean percentage of larvae at 32 and 46 dph displaying different degrees of damage (normal = white bars, with shading increasing with increasing severity of damage) at three different treatment levels (C = control, M = medium and H = high treatment) for each of the different tissues. Below: damage key. Vacs = vacuoles. N= 16-27 larvae per replicate. ($N_{C32}= 26$, $N_{C46}=27$; $N_{M32}= 24$, $N_{M46}=19$; $N_{H32}= 16$, $N_{H46}= 24$). For statistical tests, see Supplementary Tables 2 and 3.

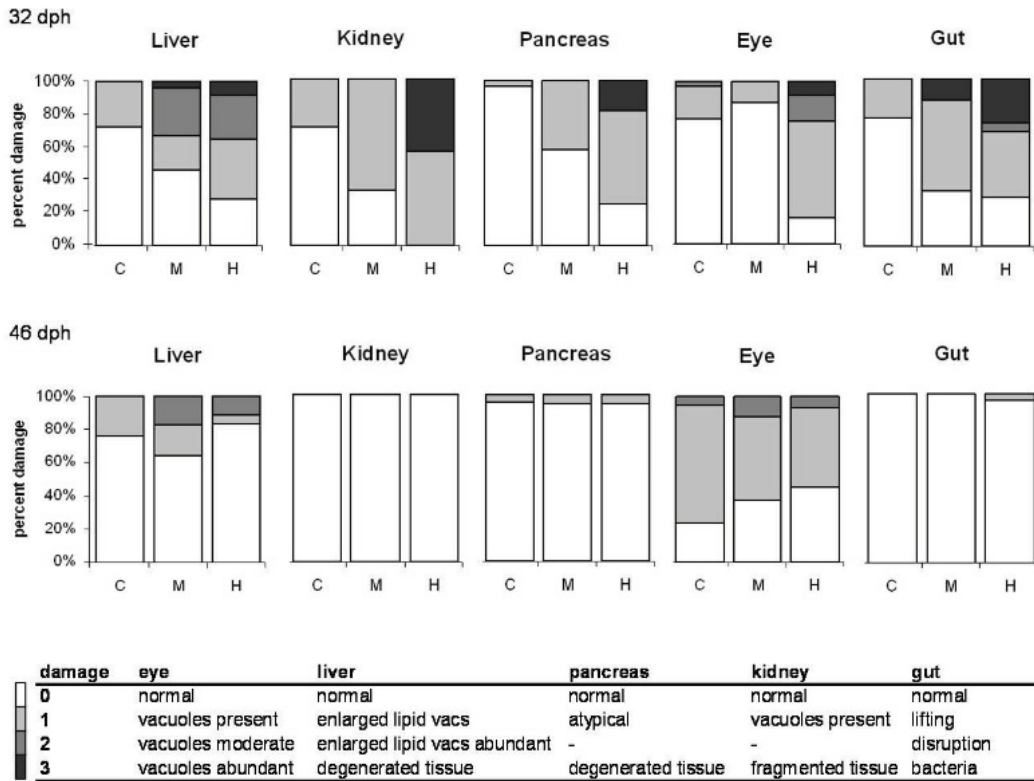
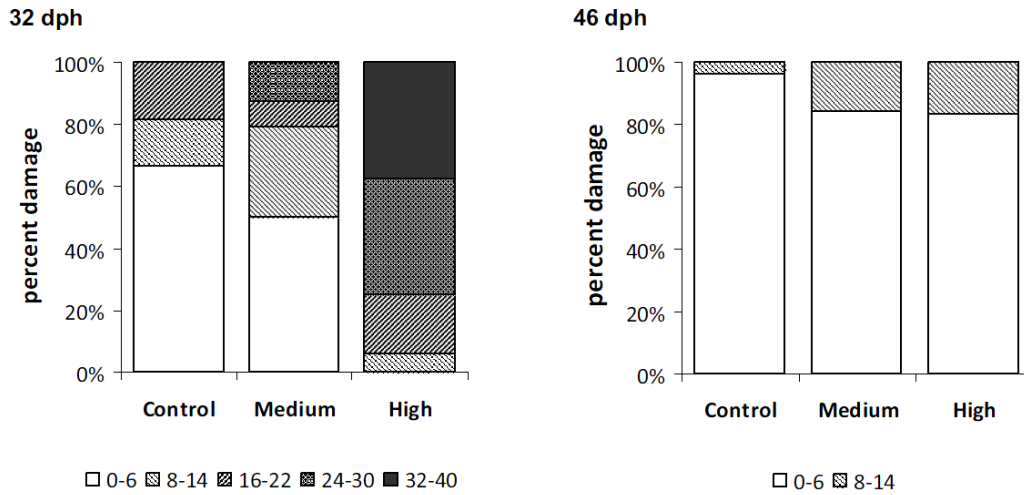


Figure 5: Quantification of total damage to larvae at increasing $p\text{CO}_2$. Percentage of larvae displaying different degrees of total damage (calculated by the total index *Tot-I*) at 32 and 46 dph and three different treatment levels (control, medium and high treatment). Normal = white bars, with shading increasing with increasing severity of damage blocked into 5 categories.



Discussion

Discussion

Variability in otolith calcification under elevated $p\text{CO}_2$ conditions

The results on otolith growth of Atlantic cod and herring larvae under elevated $p\text{CO}_2$ revealed that ocean acidification affects otolith calcification of both species. However, the direction of the response was species-specific with increased otolith growth in cod and decreased otolith growth in herring. As described previously, the precipitation of CaCO_3 into otoliths is governed by the aragonite saturation state, specifically high concentrations of Ca^{2+} and CO_3^{2-} under relatively high pH conditions, and in the presence of organic and other ionic precursors (Payan et al. 2004; Tohse and Mugiya 2008). Under elevated $p\text{CO}_2$ stress, the organism's ability to maintain high pH in the extracellular spaces such as the endolymph will influence greatly the rate of otolith calcification. In juvenile and adult fish, the maintenance of high pH under elevated ambient $p\text{CO}_2$ is carried out by raising the concentration of HCO_3^- in blood and extracellular spaces in exchange for Cl^- through a branchial acid-base ion transfer (Brauner and Baker 2009). About 90% or more of the net acid-base relevant ion transport in fish is carried out by the gills during compensation of the acid-base balance (Brauner and Baker 2009). However, functional gills are not yet fully developed in the larval Atlantic cod and herring considered here. In cod, structures that promote effective branchial respiration begin to develop 40-50 days after hatching (Hunt von Herbing et al. 1996) while gill respiration in herring becomes important when gill area has increased at a body length of about 25 mm (Batty 1984). However, fish larvae are not totally vulnerable against fluctuations in acid-base balance. Osmoregulation and acid-base regulation are also carried out by cutaneous respiration throughout the embryonic and larval fish stages (Hunt von Herbing et al. 1996; Rombough 1998; Marshall and Grosell 2005).

The differences in the efficiency of acid-base regulation through the skin between Atlantic cod and herring larvae could have contributed to the differences in the growth of otoliths. Accessory respiratory structures such as the pseudobranch develop very early in the ontogenetic development of cod larvae (Hunt von Herbing et al. 1996). The pseudobranch is a structure positioned in the head of cod larvae and contains large volume of blood cells and thin epithelial lining for gas exchange and osmoregulation (Hunt von Herbing et al. 1996; Matthey et al. 1978). Pigmented haemoglobin in the red blood cells that aids in more efficient respiration also form early in development of cod larvae (Hunt von Herbing et al. 1996). Pigmented red blood cells are important for the

acid-base regulation because of the presence of $\text{Cl}^-/\text{HCO}_3^-$ exchangers and carbonic anhydrase in the plasma membrane and cytosol of red blood cells, respectively (Marshall and Grosell 2005). In contrast, early stages of herring larvae seem to have less efficient acid-base regulation when confronted with hypercapnic stress due to the absence of accessory respiratory structures. Herring larvae do not possess respiratory pigments possibly causing less regulatory mechanism for gas exchange in larvae prior to metamorphosis (De Silva and Tytler 1973). The development of the circulatory system and respiratory vascular apparatus also happen later in the development of herring larvae while the overall surface of the body functions as a respiratory organ until the formation of the functional gills (Soin 1971). This could become problematic for herring larvae in high ambient $p\text{CO}_2$ since the absence of other accessory respiratory and osmoregulatory structures such as the pseudobranch and pigmented red blood cells means they may have a less efficient acid-base regulation system. In addition, the elongated shape of herring larvae could provide greater surface area-to-volume ratio for diffusion of CO_2 into the larvae since dissolved CO_2 is freely permeable across biological membranes (De Silva and Tytler 1973; Marshall and Grosell 2005). Ionic regulation in herring larvae is mediated by cutaneous chloride cells, which are non-uniformly distributed in the different regions of the skin and in association with the haemocoel or primordial blood vessels (Wales and Tytler 1996). However, the pseudobranch in cod larvae is more effective in ionic transport and regulation than the chloride cells for the following reasons: 1) greater association of mitochondria and tubule membrane in the pseudobranch than in chloride cells, 2) more elaborate organization of the pseudobranch, which is possibly related to carbonic anhydrase production, and 3) rich innervation of the pseudobranch suggesting some degree of neural control mechanism of osmoregulation (Mattey et al. 1978). It could also be possible that the energy needed for efficient cutaneous acid-base regulation in herring larvae was not met considering the probable rechanneling of energy for growth. Herring larvae from the elevated $p\text{CO}_2$ groups were significantly shorter and lighter than the larvae from the control group. Energy for pH compensation could have been reallocated for the needed growth while pH is maintained to a minimum required to deal with acidosis. In fishes, a bicarbonate concentration threshold puts a limit to their effective extracellular pH regulation through $\text{HCO}_3^-/\text{Cl}^-$ exchange (Brauner and Baker 2009). The specific growth rates of herring larvae both in length and weight from 32 to 39-dph showed that the larvae from the elevated $p\text{CO}_2$

groups were catching up on the growth rates of the control group even though they remained significantly smaller.

Nonetheless, the magnitude and direction of otolith fluctuating asymmetry were not affected by elevated $p\text{CO}_2$. It can be inferred that the carbonate chemistry and the conditions for otolith growth in the endolymph did not differ significantly between the left and right sides of the fish.

Putting the results into perspective, the response to elevated $p\text{CO}_2$ of the otolith growth in Atlantic cod and herring larvae confirms the variable responses observed in a wide-range of calcifying marine organisms. Increased otolith growth in white seabass larvae with increase in $p\text{CO}_2$ was previously reported by Checkley et al. (2009). Franke and Clemmesen (2011) previously reported that Baltic herring reared under elevated $p\text{CO}_2$ from fertilization until hatching, showed a significant increase in the area of the lapillus, but not the sagittal, with no corresponding significant effects in fish length and weight. Munday et al. (2011a) also found larger otoliths in clownfish, *Amphiprion percula*, reared in 1721 $\mu\text{atm } p\text{CO}_2$ but not in 1050 μatm relative to larvae in the control group. On the other hand, Frommel et al. (2012b) reported no significant effects of $p\text{CO}_2$ on the growth of the lapillus in Baltic cod reared from fertilized eggs until early non-feeding larval stages.

Resilient swimming behavior under elevated $p\text{CO}_2$ conditions

In general, the swimming behavior of both Atlantic cod and herring larvae were not significantly affected by elevated $p\text{CO}_2$. The stop duration, which refers to the amount of time the larva is not actively swimming, seemed to be the most sensitive kinematic variable to elevated $p\text{CO}_2$ concentrations with significant subtle changes observed, i.e. shorter stops for cod larvae and longer stops for herring larvae. The implications of these subtle changes to the larvae in our studies are unknown. However, based on the functionality of the stop duration, shorter stop time for cod larvae could mean less time spent searching for food (O'Brien et al. 1989; Galbraith et al. 2004) while longer inactive periods in herring larvae could increase their sinking rate (Batty 1987). The changes - if biologically significant - could affect the ability of the larvae to search for food, escape from predators, or ability to prevent sinking.

In studies where the direct ecological implications of larval behavior in response to elevated $p\text{CO}_2$ were investigated, significant effects were reported. The effects include damage to olfaction with impact on homing ability (Munday et al. 2009b) and detection

of prey and predator (Munday et al. 2010; Dixson et al. 2010; Cripps et al. 2011), and damage to brain and neurotransmitter functions (Domenici et al. 2012; Nilsson et al. 2012; Ferrari et al. 2012). Although the ecological end points of the larval behavior were significantly affected, the details of the swimming kinematics of these larvae could have remained robust to $p\text{CO}_2$ increase. In other words, a larva might prefer to swim towards a predator instead of avoiding it but the swimming patterns itself are not significantly affected.

Plasticity in the functional morphology of otoliths

The thesis was undertaken to investigate the link between the structure of the otoliths and their associated mechanoreceptor functions for maintaining balance, detection of movement and acceleration in fish larvae. Although hearing is an important function performed by otoliths, the thesis did not include investigation on the acoustic functionality because it requires more specialized observation procedure. The changes in the associated functions were inferred from the kinematics of the swimming patterns of Atlantic cod and herring larvae. The functional morphology of the otoliths was investigated using the ocean acidification scenarios arising from anthropogenic emission of CO_2 as the external driving factor to elicit changes in the otolith structure and swimming behavior. Although severe tissue damage arising from hypercapnia was reported at specific age groups in our study, i.e. at 32-dph for Atlantic cod larvae (Frommel et al. 2012) and at 25-dph for herring larvae based on preliminary results, observations of behavior and otolith growth in other age groups without severe tissue damage provided the window to observe functional morphology of the otolith. Thus, the potential effects of elevated $p\text{CO}_2$ on the morphology of the otoliths and the associated behavioral changes were not masked by tissue damages that could also affect the swimming behavior of the larvae. In the case of cod larvae, the behavioral observations were made before the onset of tissue damage while in herring larvae, the observations were made after the event. It should be noted, however, that the smaller herring larvae observed in the elevated $p\text{CO}_2$ treatments at 32 and 39-dph could represent the survivors of the mortality resulting from tissue damages and may have a different set of behavior. However, comparison of the swimming kinematics of the smaller larvae from the elevated $p\text{CO}_2$ treatment at 40-dph to similar-sized larvae from the control treatment at 34-dph revealed only subtle differences in swimming kinematics.

The results of the study confirmed that the otoliths of Atlantic cod and herring larvae significantly responded to perturbations in the ambient seawater carbonate system, simulating the consequence of ocean acidification. Variations in the ability of the organism to regulate the extracellular pH could have influenced the direction of the response in otolith growth. On the other hand, results showed that the structural changes in the otoliths did not significantly translate into changes in the swimming behavior of the larvae. The magnitude of the changes in otolith growth could be within the variations resulting from other external factors such as changes in temperature and food availability that would still maintain the mechanoreception functionality of the otoliths (Folkvord et al. 2004; Neuman et al. 2001; Otterlei et al. 2002). Based on the concept of symmorphosis, which is a hypothesis for examining structure-function relationships in organisms including fish, biological structures should match but not exceed the functional requirements of the organism (Rombough and Moroz 1997; Weibel et al. 1991). Thus, it can be said that the growth of the otoliths under an acidified environment could deviate from the optimum structural design expected under normal $p\text{CO}_2$ conditions. However, the deviations are still within the variations required for the effective functioning of the otoliths as organs for maintaining balance and movement detection, thereby still rendering normal swimming behavior in both Atlantic cod and herring larvae.

The changes that will take place in the oceans towards the end of the century and beyond also involve other factors such as increasing water temperatures and expansion of hypoxic zones. The CO_2 problem is not confined to ocean acidification alone. The maintenance of the functionality of the otoliths despite the change in growth as concluded in the thesis will most likely be challenged more by the synergistic effects of $p\text{CO}_2$ with other factors such as temperature and oxygen levels.

Outlook: Multistressor (Temperature, $p\text{CO}_2$) effects on otolith calcification

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Description: This section provides information on the effects of two factors, temperature and $p\text{CO}_2$, on the growth of otoliths of juvenile Atlantic cod, *Gadus morhua* L., from three populations namely, Norwegian coastal cod-Trondheim, North Sea cod-Helgoland, and North Sea cod-Sweden. The juveniles were incubated for at least two months in two temperature regimes (10 and 18°C) and at least two $p\text{CO}_2$ concentrations (Trondheim: 380 and 1900 μatm ; Helgoland: 380 and 2400 μatm ; Sweden: 380, 1100, 2400 μatm). The focus of this study was to check whether temperature and $p\text{CO}_2$ have synergistic effects on the growth of the cod juvenile otoliths. The hypothesis of a constant otolith area/sulcus area ratio was also investigated in these samples because in juvenile otoliths, the sulcus region is more distinguishable compared to the larval otoliths. The sulcus area/otolith area ratio provides an approximation on the number of hair cells that articulate with the otolith, which has implications on the mechanoreceptor function of the otoliths (Torres et al. 2000; Lychakov and Rebane 2000; Popper et al. 2005).

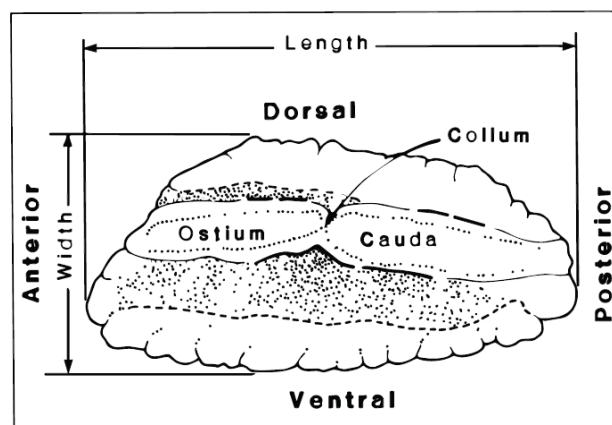


Figure 1. Diagram of an otolith showing the sulcus area, which is comprised of ostium and cauda regions (from Hunt 1992).

Table 1. Summary of the results of the statistical analysis (Factorial ANOVA) conducted to compare the responses of the different variables to different combinations of temperature and $p\text{CO}_2$ concentrations. Measurements for otolith and sulcus area for the Swedish samples are not yet completed. (Symbols: + increase, - decrease, = no effect of main factor, NI no interaction between temperature and $p\text{CO}_2$).

| Variables | Trondheim | | | Helgoland | | | Sweden | | |
|-----------------------------|-----------|----------------|------------------|-----------|----------------|------------------|--------|----------------|------------------|
| | T | $p\text{CO}_2$ | TxpCO_2 | T | $p\text{CO}_2$ | TxpCO_2 | T | $p\text{CO}_2$ | TxpCO_2 |
| Oto area / fish length | + | = | + | = | = | NI | | | |
| Oto area / fish weight | + | = | NI | + | + | NI | | | |
| Oto weight / fish weight | + | = | NI | + | = | NI | + | = | NI |
| Ostium/Caudal area ratio | = | = | NI | = | = | - | | | |
| Sulcus/Otolith area ratio | = | + | + | = | = | NI | | | |
| Sulcus/Otolith weight ratio | = | + | + | = | + | NI | | | |

Analysis:

1. Temperature increase had more significant influence on otolith area and weight compared to increase in $p\text{CO}_2$. Wider and bigger otoliths resulted from higher temperatures in the three cod populations.
2. There were no synergistic effect of temperature and $p\text{CO}_2$ concentrations on otolith area and weight.
3. There was a population-specific response of the sulcus area/otolith area and sulcus area/otolith weight ratio. For the Norwegian coastal cod-Trondheim population, an increase in sulcus area/otolith area ratio and sulcus area/otolith weight ratio resulted from the $p\text{CO}_2$ increase and from the synergistic effects of temperature and $p\text{CO}_2$. At higher temperature, the influence of $p\text{CO}_2$ on sulcus area/otolith area ratio was enhanced.

Implication:

Sulcus area/otolith area ratio is highly conserved i.e. species and habitat-specific (demersal vs pelagic) (Lombarte 1992; Aguirre and Lombarte 1999; Lychakov and Rebane 2000; Torres et al. 2000). It has been shown in this study that certain population of Atlantic cod might experience decoupling of the optimum proportion of the sulcus and otolith areas.

Acknowledgements

Vielen Dank! Tussen Takk! Thank you! Maraming Salamat!

To the European community for funding my PhD project through the Marie Curie Initial Training Network CalMarO Project. I hope Europe can ride out the current economic crisis to be able to continue promoting value for education and advancement of scientific knowledge.

To my CalMarO supervisors Catriona, Uwe and Audrey for guiding my scientific career and the assistance to make my family's stay in Kiel and Bergen very comfortable. To Arild for continuing to guide me even after my master thesis and giving me more lessons in statistics.

To my co-CalMarO fellows (Reidar, Frauke, Holger, Mei, Vincent, Luke, Fred, Socrates, Marlene, Noa, and Cristina), for the nice fellowship and the wonderful memories every time we met for a workshop. To Nicole, Avan, Alexandra, Skadi and Jörg for the efficient management of CalMarO and for setting up the useful workshops. To ISOS for the valuable trainings and workshops that made my PhD experience more holistic and multidisciplinary.

To Frank Melzner and the whole group (Magda, Marian, Katja, Jörn, Uli, Meike, Isabel, Rainer, Chico) for giving me my first circle of friends in Germany even before I got the fellowship. Thank you for helping me set up my first adventures with the exciting cuttlefish.

To the Espegrend team (Andrea, Gladys, Catriona, Audrey, Arild, Mei) who labored night and day for the success of the mesocosm experiment. To the assistance provided by Oddbjørn, Vibeke, Knut, Agnes, Frank Midtøy and Tomas, without which the Espegrend experiment would have been much more difficult. To the expert services provided by Richard Bellerby, Hildegunn Almelid, and Jane Strømstad in analyzing our water samples for the experiment. To Catriona for driving all the way from Kiel to Espegrend passing through underwater fjord tunnels just to assist us in the experiment. Icelandic volcanic eruption was not enough to stop you from joining us.

To the Austevoll larval behavior team (Caroline, Reidun, Yuichi, Anne Berit) led by Howard for giving me the opportunity to pursue the other part of my thesis, which is the behavioral aspect of the otolith functional morphology. To Howard and Anne Berit for picking me and Gladys up at the Espegrend station very early in the morning and giving us a lift to Austevoll with all the water containers and for the nice discussions aboard the Krokeide-Hufthamar ferry. To Caroline for the big help in doing the statistical analysis. To Roberto Racca for fixing the TrakFish and Anapath softwares all the way from Canada.

To the administration personnel in Geomar and UiB for the efficient processing of my papers.

To my Hiwis (Lars+1, Kim, Burkhard, Ashlie, Sophie, Christian, Katharina) who helped me with the preparation of my samples. To Julia who helped me in dissecting the herring otoliths and in rearing of hake larvae.

To my colleagues in Geomar (especially to Helgi, Nina Garilao, Nina Bergmann, Sally Degg, Sven, Rudi, Albert) and UiB, thank you for the company and assistance.

To my other scientific collaborators who encourage me to think in a multidisciplinary way to address interesting questions in my PhD thesis. Apologies for not being able to include some of the works in my dissertation:

To Stefan, Hannah and Tina Treude for allowing me to use the microsensors and additional work on electron microscopy.

To Rajan and his lab for doing the proteomics analysis of the herring and cod larvae.

To Katharina Michael, Cornelia Kreiss and their team for sending me juvenile cod otolith samples that provided me opportunity to look into the sulcus:otolith area relationship.

To Dorrit Jacob and Ursula Wehrmeister for analysing the calcium carbonate polymorphs of the otolith samples using Raman spectroscopy.

To Mario Thöner for helping me with the microchemistry analysis of otolith and statolith samples using the wavelength dispersive x-ray spectroscopy.

To Ana Kolevica and Anton Eisenhauer for helping me with the microchemistry analysis of my water samples.

To Hagen Vöhrs for checking the possibility of using dental techniques from the UniKlinik to analyze my otolith samples.

To my sisters and their families in Butuan and to my in-laws in Los Baños for the constant communication, which made our gloomy winter bearable.

To Papa Rolly and Mama Mely for the sacrifices to support my education. Reaching my PhD will never be possible without the foundation you gave me.

And most especially to my dearest wife, Gladys, for giving up her career in the Philippines to follow and support me. Thank you for acting as my ultimate research assistant as we travel back and forth to Austevoll and to the High Technology Center. Europe is a much more enjoyable and memorable place to go around with you by my side. And with our little Nolan Alaric who gave me an extension for my PhD, our family is much more complete.

And to the God of my faith, thank you for giving me the strength and wisdom to act accordingly as I travel my journey. I hope you will continue to guide me as I seek scientific knowledge to fight ignorance and intolerance around me.

Kiel, September 11, 2012

Rommel H. Maneja

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Description of author contributions

Chapter I

Effects of ocean acidification on the calcification of otoliths of larval Atlantic cod,

Gadus morhua L.

(for resubmission in Marine Ecology Progress Series)

Authors: RH Maneja, AY Frommel, AJ Geffen, A Folkvord, U Piatkowski, MY Chang
and C Clemmesen

Contributions: RHM, AYP, AJG, AF, UP, CC designed the experiment; RHM, AYP, AJG, AF, CC, MYC performed the experiment; RHM dissected the otoliths and generated data from the otolith samples; RHM, AF, AJG, CC analyzed the data; RHM wrote the paper. All authors contributed to the discussion of the results and in the editing of the manuscript.

Chapter II

The swimming kinematics of larval Atlantic cod, *Gadus morhua* L., are resilient to elevated seawater $p\text{CO}_2$

(published in 2012 Marine Biology Special Issue on Ocean Acidification)

Authors: RH Maneja, AY Frommel, HI Browman, C Clemmesen, AJ Geffen,
A Folkvord, U Piatkowski, CMF Durif, R Bjelland and AB Skiftesvik

Contributions: RHM, AYP, CC, AJG, AF, UP designed the experiment; RHM, AYP, CC, AJG, AF performed the experiment; RHM, HIB, CMFD, RB and ABS performed the video recording of the larval swimming behavior; RHM processed and extracted data from the video recordings; RHM, CMFD, HIB analyzed the data; RHM wrote the paper. All authors contributed to the discussion of the results and in the editing of the manuscript.

Chapter III

Effects of elevated $p\text{CO}_2$ on the swimming kinematics and foraging behavior of larval Atlantic herring, *Clupea harengus* L.

(manuscript in preparation)

Authors: RH Maneja, AY Frommel, HI Browman, AJ Geffen, A Folkvord,
U Piatkowski, CMF Durif, R Bjelland, AB Skiftesvik and C Clemmesen

Contributions: RHM, AYF, AJG, AF, UP, CC designed the experiment; RHM, AYF, AJG, AF, CC performed the experiment; RHM, HIB, CMFD, RB and ABS performed the video recording of the larval swimming behavior; RHM processed and extracted data from the video recordings; RHM, CMFD, HIB analyzed the data; RHM wrote the paper. All authors contributed to the discussion of the results and in the editing of the manuscript.

Chapter IV

Severe tissue damage in Atlantic cod larvae under increasing ocean acidification
(second author, published in Frommel et al. 2012. Nature Climate Change 2:42-46)

Authors: AY Frommel, RH Maneja, D Lowe, AM Malzahn, AJ Geffen, A Folkvord,
U Piatkowski, TBH Reusch and C Clemmesen

Contributions: AYF, RHM, AJG, AF, UP and CC designed the experiment; AYF, RHM, AJG, AF and CC performed the experiment; DL performed the histological analysis and wrote the section on that topic; AMM performed the lipid analysis; AYF, CC and TR analyzed the data; AYF and TR wrote the main paper. All authors discussed the results and implications and commented on the manuscript at all stages.

Declaration

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I hereby declare

1. that apart from the supervisors' guidance, the content and design of the essay is all my own work;
2. that the thesis is my first doctoral thesis and has not been submitted either partially or wholly as part of a doctoral degree to another examining body;
3. that Chapter I of the thesis is for resubmission after first round of review to the Marine Ecology Progress Series journal; Chapter II of the thesis was published in 2012 Marine Biology Special Issue on Ocean Acidification; Chapter III is manuscript in preparation; Chapter IV was published in Nature Climate Change journal and has been included in the doctoral dissertation of the first author of the paper; and
4. that the thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation.

Kiel, October 24, 2012

Rommel H. Maneja