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

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ABSTRACT: The growth and development of the aragonitic $CaCO_3$ otoliths of teleost fish could be vulnerable to processes resulting from ocean acidification. The potential effects of an increase in atmospheric CO_2 on the calcification of the otoliths were investigated by rearing Atlantic cod *Gadus morhua* L. larvae in 3 pCO_2 concentrations — control (370 µatm), medium (1800 µatm) and high (4200 µatm) — from March to May 2010. Increased otolith growth was observed in 7 to 46 d post hatch (dph) cod larvae at elevated pCO_2 concentrations. The sagittae and lapilli were usually largest in the high pCO_2 treatment followed by the medium and control treatments. The greatest difference in mean otolith surface area (normalized to fish length) was for sagittae at 11 dph, with medium and high treatments being 46 and 43 % larger than the control group, respectively. There was no significant pCO_2 effect on the shape of the otoliths nor were there any trends in the fluctuating asymmetry, defined as the difference between the right and left sides, in relation to the increase in otolith growth from elevated pCO_2.

KEY WORDS: 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 CO_2. This is due to higher solubility of CO_2 at low water temperatures, increased air–sea exchange as sea-ice loss increases from global warming, CO_2 remineralization of high organic carbon loads from seasonal primary production and low-alkalinity riverine inputs (Fabry et al. 2009). Upwelling events in coastal waters could enhance the levels of ocean acidification by bringing deeper acidic waters with remineralized CO_2 to the surface, 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, the largest cod population in the world, is located around the Lofoten archipelago in Norway, including Vestfjorden, which is also subject to wind-driven upwelling and downwelling events (Furnes & Sundby 1981). Upwelling caused by winds blowing from the southwest spreads the eggs out toward the central part of Vestfjorden, while downwelling due to winds from the northeast concentrates the older egg stages toward 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-pCO_2 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).

Teleost fishes produce otolith structures that are mainly composed of aragonitic $CaCO_3$ (Degens et al. 1969), which may be sensitive to elevated levels of pCO_2. Munday et al. (2011a) discussed 3 possible consequences of ocean acidification on otolith calcification. First, fish might have more difficulty in precipitating aragonite as the available carbonate ion concentration declines in seawater. Second, otolith hypercalcification 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 pCO_2. Third, physiological stress from acidic conditions could indirectly influence otolith shape and symmetry. Otolith hypercalcification was reported in white sea bass larvae *Atractoscion nobilis* at 993 and 2558 µatm CO_2 (Checkley et al. 2009) and clownfish larvae *Amphiprion percula* at 1721 µatm (Munday et al. 2011a). In contrast, otolith calcification was not affected in spiny damselfish *Acanthochromis polyacanthus* juveniles grown at 850 µatm CO_2 (Munday et al. 2011b).

In the present study, the influence of elevated seawater pCO_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, Lombarte 1992, Aguirre 2003), 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 land-based mesocosms at the University of Bergen's Espegrend Marine Station from March to May 2010. Computer-controlled bubbling of CO_2 into the bottom of nine 2650 l experimental tanks was done to achieve 3 pCO_2 levels: control (370 µatm), medium (1800 µatm) and high (4200 µatm), with 3 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 experimental tanks inside 2 water baths, which minimized the influence of fluctuations in air temperature. The gradual rise in temperature reflected the natural increase in water temperature of the fjord at 40 m depth, which was the source of the water with mean salinity of 33.3 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 Norwegian Institute of Marine Research were transferred to the floating incubation buckets in the experimental tanks on March 25, 2010. The eggs remained under normal pCO_2 conditions for 3 d, after which the pCO_2 levels were adjusted to the targeted pCO_2 for each treatment. Fifty percent hatching occurred on April 9, 2010, and that date was designated as 0 d 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 a Hydrotech size-selective filter system (Seljeset et al. 2010). The larvae were initially fed with zooplankton with a size fraction of 80–250 µm and this was 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 8 times from 7 dph until 46 dph (at 7, 11, 14, 18, 25, 32, 39 and 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 to 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 <30 s and frozen individually in Eppendorf tubes at −80°C. Standard length (SL) of the fish larvae was measured using the calibrated pictures. The

lapilli and sagittae were dissected from the fish larvae using fine needles and mounted on glass slides using CrystalbondTM 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 (1000× magnification), while larger otoliths from older larvae were taken at 200 to 600× magnification. The otolith surface area and dimensions were measured from the pictures of the otoliths using ImageJ software (Schneider et al. 2012).

Analysis

The otolith surface area was compared among treatments using analysis of covariance (ANCOVA), with fish SL 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 of the larvae in the 3 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 2 age groups (32 and 46 dph) to meet the ANCOVA assumption of homogeneity of regression slopes among the 3 pCO_2 treatments, which is required to test for intercept (otolith area) differences. Regression slopes among treatments were not significantly different in the rest of the age group.

In Atlantic cod, an ontogenetic shift in the growth rates of the sagitta and lapillus takes place, with larger lapilli at the earlier larval stages and then a shift to larger sagittae in later stages. Bergstad (1984) reported that the shift in the growth rates of sagitta and lapillus occurred at ~25 dph or 6 mm SL. This transition was monitored in the present study by tracking changes in the ratio of the surface area of the sagitta to that of the lapillus for each fish larva. The sagitta/lapillus surface area ratios were compared among pCO_2 treatments per sampling date using nested ANOVA, with tanks nested in pCO_2 treatments. A non-parametric Kruskal-Wallis test was performed for those sampling dates when the sagitta/lapillus surface area ratios did not satisfy assumptions for normality and homogeneity of variance. The same procedure for nested ANOVA and Kruskal-Wallis test was performed on fish SL data.

Differences in otolith surface area between the right and left sides, referred to as fluctuating asymmetry (Somarakis et al. 1997), were derived for each larva when both right and left otoliths were avail-

able. Higher variability was hypothesized in the high CO_2 treatment. The differences between the 2 sides were normalized by dividing by the mean otolith surface area of the 2 otoliths and then plotted against age of the larvae. The homogeneity of variances of the fluctuating asymmetry with age among the pCO_2 treatments was tested using Hartley F-max tests of homogeneity of variances.

The shape of the lapilli and sagittae was also compared using the roundness parameter derived from the otolith pictures using ImageJ. A value of 1 means a perfect circle, while values approaching 0 indicate an increasingly elongated shape (Schneider et al. 2012). A nested ANOVA was used to compare otolith roundness among the pCO_2 treatments with replicate tanks nested in each treatment. The individual roundness value from both the left- and right-side otoliths was included in the analysis.

RESULTS

Increases in the growth of both lapillus and sagittal otoliths were observed in 7 to 46 dph cod larvae (Fig. 1). Larger otolith surface areas were observed in the high, followed by the medium and control treatments. The otolith surface area of larvae in the medium treatment fluctuated between values of the control and high treatments. The greatest 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 % larger than the control group, respectively.

An ontogenetic shift in the growth rates of the lapilli and sagittae was observed in all larvae (Fig. 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 compared to the larvae from control and medium treatments. At 11 and 18 dph, the high treatment larvae already showed significantly higher sagitta/lapillus surface area ratios than those in the control and medium treatments even though the shift in growth rates had not yet occurred. At 32 dph, the larvae in the high treatment were >6 mm SL and significantly longer than larvae in the control and medium treatments (Fig. 3). This trend of SL with increasing pCO_2 was seen at 11, 25 and 32 dph.

The left- and right-side otoliths often differed in size (Fig. 4), but there was no bias to indicate directional asymmetry or increase in the magnitude of the
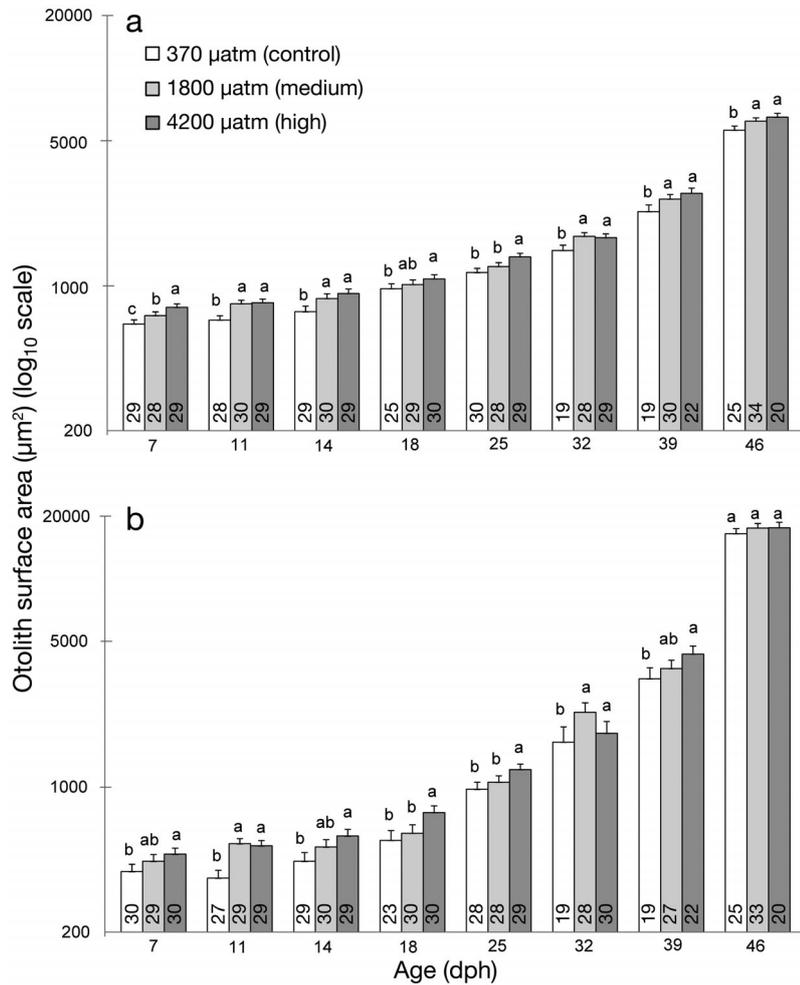
Fig. 1. *Gadus morhua*. Mean (ANCOVA-adjusted) surface area of (a) lapilli and (b) sagittae (both normalized to fish standard length [SL]) from Atlantic cod larvae grown in 3 pCO_2 treatments. Whiskers denote 95% CI. Different letters above bars denote significant differences between pCO_2 treatments based on ANCOVA test with SL as the covariate (at 5% significance level). Numbers inside bars indicate sample sizes; dph: days post-hatch

fluctuation. Variances of the differences in otolith area between right and left sides with age were homogenous amongst pCO_2 treatments (Hartley F-max: lapillus 1.28, sagitta 2.33, $p > 0.05$).

The mean roundness values of the lapillus from 7 to 39 dph ranged from 0.91 to 0.94 and did not differ among the pCO_2 treatments (nested ANOVA, $p > 0.05$) (Fig. 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 a 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 to 32 dph but became more elongated again at 46 dph. There was

no clear pCO_2 treatment effect on the shape of the sagitta except at 25 dph, when more rounded sagittae were associated with increased pCO_2 (nested ANOVA, $p = 0.016$).

DISCUSSION

Our results show that otoliths of Atlantic cod larvae (7 to 46 dph) were larger in elevated pCO_2 conditions. Increase in otolith growth began early in development (7 dph). Several studies have already explored the possibility of enhanced calcification under elevated pCO_2 conditions in marine organisms with active acid/base regulatory mechanisms. High ambient pCO_2 could result in respiratory acidosis where extracellular pCO_2 rises and pH decreases (Melzner et al. 2009). In organisms that regulate acid–base balance, 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 (Marshall & Grosell 2005, Melzner et al. 2009). Cuttlefish minimized the decrease in extracellular blood pH, decreasing by only 0.18 units, when subjected to acute hypercapnia at 0.60 kPa pCO_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 8 h and stabilized at 10.4 mM after 24 h. Similarly, Atlantic cod (230 to 525 g) showed an increase of 21 mM HCO_3^- in the blood over 24 h 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 pCO_2 conditions (Gutowska et al. 2008, 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 & Takahashi (1985) reported higher pH and total CO_2 in the endolymph compared
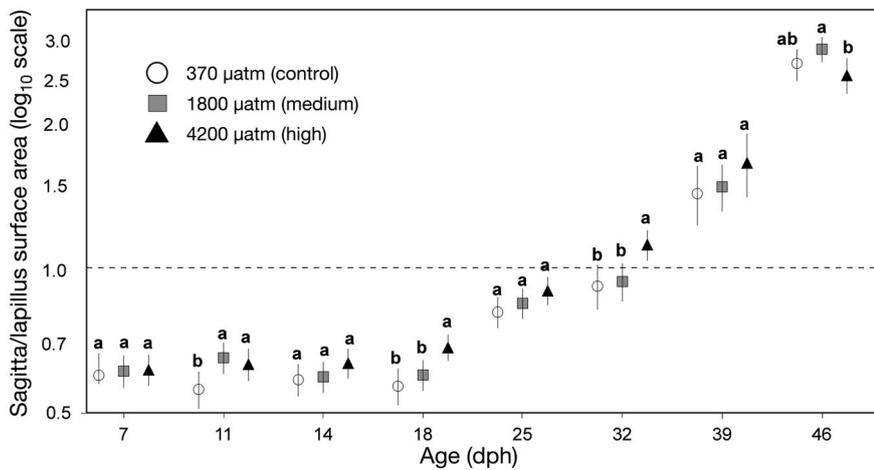
Fig. 2. *Gadus morhua*. Sagitta/lapillus surface area ratio of otoliths from cod larvae grown under 3 pCO_2 concentrations. Whiskers denote 95% CI. Different letters above whiskers denote significant differences between pCO_2 treatments based on nested ANOVA or Kruskal Wallis test, if necessary (at 5% significance level)
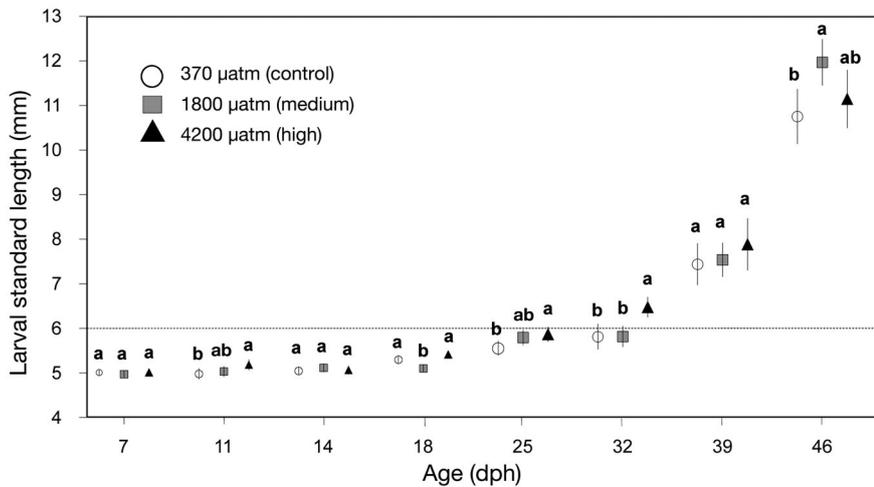
Fig. 3. *Gadus morhua*. Standard lengths of cod larvae grown under 3 pCO_2 concentrations. Whiskers denote 95% CI. Different letters above whiskers denote significant differences between pCO_2 treatments based on nested ANOVA or Kruskal Wallis test, if necessary (at 5% significance level)

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 µatm pCO_2 (Munday et al. 2011a) and in white seabass larvae at 993 and 2558 µatm pCO_2 (Checkley et al. 2009) was attributed to pH regulation in the otolith endolymph. Payan et al. (1998) provided the evidence that at an 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 pCO_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 the younger larvae. Structures that aid in effective gill ventilation, such as the opercular bones, only start to form at 40 to 50 dph (Hunt von Herbing et al. 1996). However, the increase in otolith growth in cod larvae at elevated pCO_2 suggests 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 cause 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 a large volume of blood cells and thin epithelial lining positioned in the head of cod larvae, and pigmented hemoglobin in the red blood cells (Mattey et al. 1978, Hunt von Herbing et al. 1996, Marshall & Grosell 2005).

Otoliths are the main organs for detecting acceleration, balance and sound in fish. Increase in the growth of otoliths due to elevated pCO_2 could have consequences on the otolith functions. Huelsenbeck (2010) reported less swimming activity in white seabass larvae grown at pCO_2 2500 µatm coincident with occurrence of hyper-calcified otoliths. However, cod larvae aged 12 and 27 dph from our experiment showed only subtle changes in their swimming behavior due to increased pCO_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.
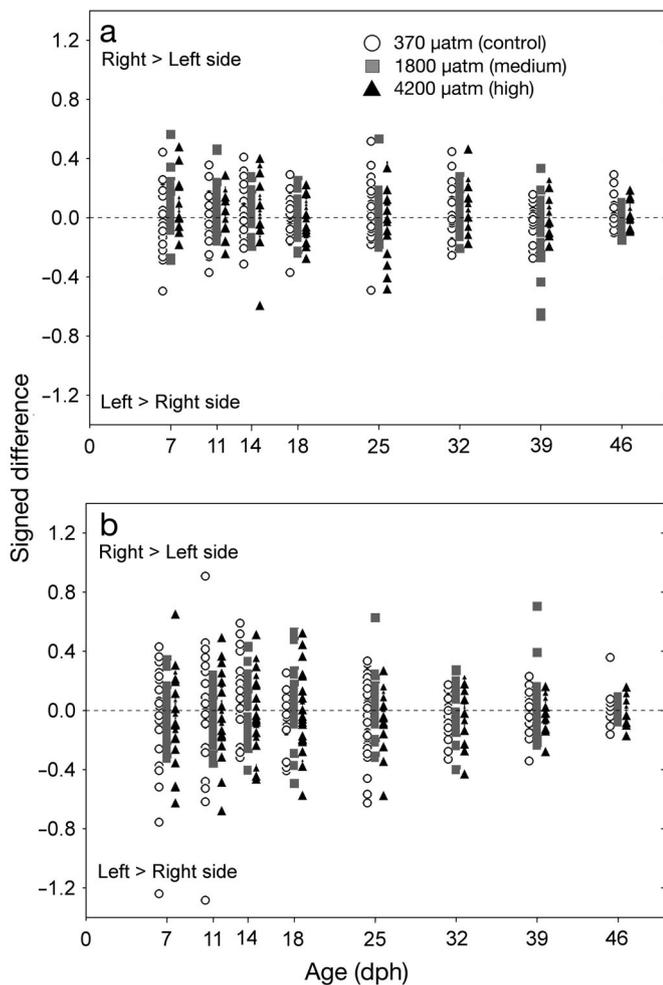
Fig. 4. *Gadus morhua*. Signed difference of otolith surface area ([right−left]/mean) of Atlantic cod larvae grown under 3 pCO₂ concentrations. Signed difference was normalized by dividing by the mean surface area of the 2 sides. Offset in the *x*-axis was used to display the data per pCO₂ treatment. (a) Lapilli, (b) sagittae

Reports on larvae of tropical marine fishes point to disruptions of neurotransmitter functions in the brain due to elevated levels of 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. 2009), loss of detection and avoidance of predators (Dixson et al. 2010, Munday et al. 2010) and loss of predatory reaction to presence of prey (Cripps et al. 2011). The impairment of the neurotransmitter function might also have affected the ability of the larvae to decide between left and right turns (Domenici et al. 2012) and caused a loss of ability to learn anti-predatory

responses (Ferrari et al. 2012). Despite the possible increase in HCO_3^- concentration in the endolymph as a consequence of pH compensation, implied from increased otolith growth with elevated pCO₂, only subtle changes in the swimming behavior of cod larvae from the same experiment were reported by Maneja et al. (2012). It is important to investigate if the disruption in the neurotransmitter functions in fish larvae due to the effects of ocean acidification (Nilsson et al. 2012) is observable only at the end-points of behavioral cue experiments (e.g. reaction to predator) and not on the specific swimming kinematic variables. For example, a fish larva that is attracted to the smell of a predator might still be swimming normally under acidified seawater conditions. It is worthwhile investigating implications of the subtle behavioral changes in cod larvae with enhanced otolith growth from elevated pCO₂ in behavioral cue experiments, such as reaction to the presence of predators and changes in food densities.

Another interesting result from the present study was the earlier transition of the growth rates of the sagittae and lapilli 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 lapilli and sagittae could have been an indirect effect of the observed differences in the somatic growth rate of the larvae. Bergstad (1984) reported that the lapilli of cod larvae reared at 5 ± 1°C were always larger than the sagittae from hatching until ~6 mm SL or at ~25 dph. Dale (1984) also reported bigger lapilli than sagittae in the early larval stage of cod. In the present study, the mean SL of the larvae from the high treatment had already surpassed 6 mm SL, and was significantly longer than the control and medium treatments, at 32 dph. Cod larvae from the high treatment also had significantly greater dry weights than fish in the control group at 32 dph, as previously reported by Frommel et al. (2012).

Although increased otolith growth was observed in both lapilli and sagittae at elevated pCO₂ levels, there was no systematic pattern of deviation from the normal fluctuating asymmetry and no severe changes in otolith shape. There was 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 an increase in otolith growth at 1721 µatm pCO₂. This
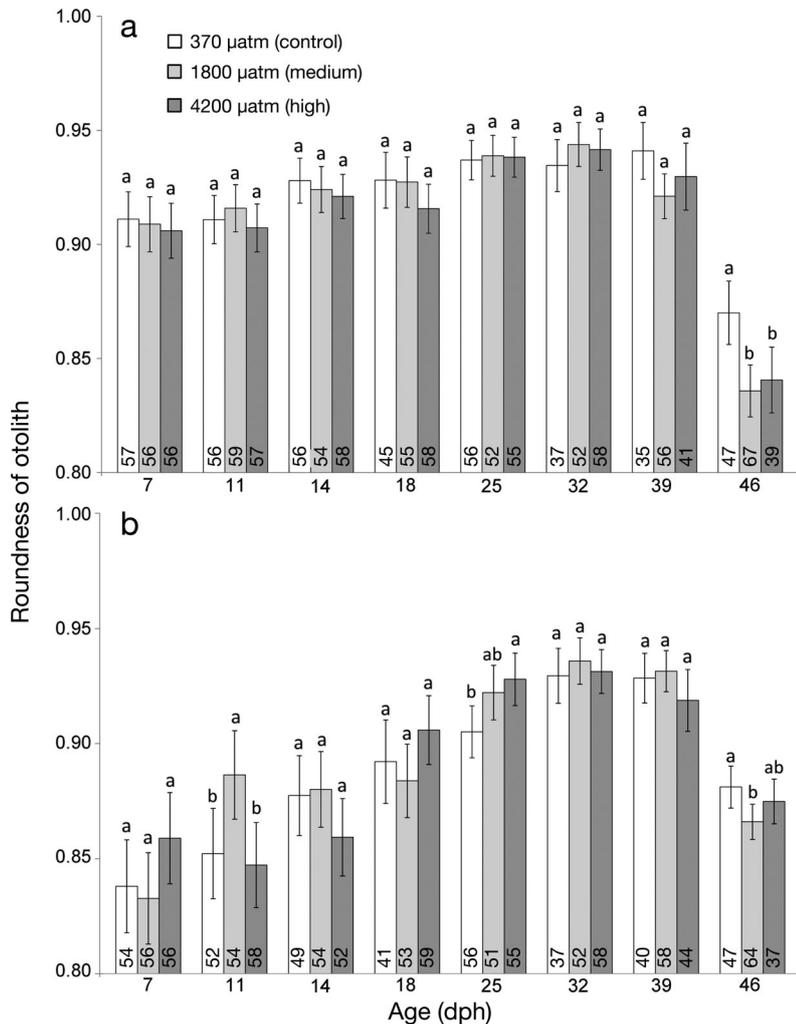
Fig. 5. *Gadus morhua*. Mean roundness of (a) lapilli and (b) sagittae from cod larvae grown under 3 pCO$_2$ concentrations. Whiskers denote 95% CI. Different letters above whiskers denote significant differences between pCO$_2$ treatments based on nested ANOVA (at 5% significance level). Roundness values range from 0 (highly elongated) to 1 (perfect circle). Numbers inside bars indicate sample sizes

temporal processing and sound localization, were shown through mathematical modeling to be affected by increasing otolith mass asymmetry, particularly those involving large otolith masses.

In summary, the present study showed that under conditions mimicking global and locally enhanced ocean acidification, otolith growth in Atlantic cod larvae increased and size-related transition in lapillus and sagittal growth rates occurred earlier than under normal conditions. However, the magnitude of fluctuating asymmetry did not increase, nor did it lead to directional bias in otolith growth or to severe damage in otolith shape. The increase in otolith calcification due to elevated pCO$_2$ might be well within the natural variation in otolith sizes maintaining normal functioning.

could also mean that changes in the carbonate chemistry inside the endolymph occur at the same magnitude in both left and right sides. The subtle deviations from bilateral symmetry in otolith size have 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 & Rebane (2005) suggested that a specific physicochemical mechanism of the paired otolith growth must exist, allowing 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,

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