LETTERS
PUBLISHED ONLINE: 11 DECEMBER 2011 | DOI: 10.1038/NCLIMATE1324
nature climate change

Severe tissue damage in Atlantic cod larvae under increasing ocean acidification

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Ocean acidification, caused by increasing atmospheric concentrations of CO_2 (refs 1–3), 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[4,5]. In teleost fishes, early life-history stages are particularly vulnerable as they lack specialized internal pH regulatory mechanisms[6,7]. So far, impacts of relevant CO_2 concentrations on larval fish have been found in behaviour[8,9] and otolith size[10,11], mainly in tropical, non-commercial species. Here we show detrimental effects of ocean acidification on the development of a mass-spawning fish species of high commercial importance. We reared Atlantic cod larvae at three levels of CO_2, (1) present day, (2) end of next century and (3) an extreme, coastal upwelling scenario, in a long-term ($2\frac{1}{2}$ months) mesocosm experiment. Exposure to CO_2 resulted in severe to lethal tissue damage in many internal organs, with the degree of damage increasing with CO_2 concentration. As larval survival is the bottleneck to recruitment, ocean acidification has the potential to act as an additional source of natural mortality, affecting populations of already exploited fish stocks.

Present average CO_2 levels in the atmosphere have already exceeded 380 ppm 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[12]. Approximately a third of excess CO_2 in the atmosphere will be dissolved in ocean waters, leading to an estimated drop in pH of 0.4 units ($pCO_2 \sim 1{,}000\,\mu atm$) globally by the year 2100 and up to 0.8 units ($pCO_2 \sim 2{,}000\,\mu atm$) by the year 2300 (refs 1–3). Locally, the effects can be even more severe, especially in coastal regions with upwelling of oxygen-poor, CO_2-rich water, and pCO_2 values above 4,000 µatm in the future could be reached in habitats where cod larvae occur[13].

The Atlantic cod, *Gadus morhua*, has a wide distribution throughout the North Atlantic Ocean. Several eastern Atlantic populations are found from the high Arctic down to North and Baltic seas, where they experience very different conditions in terms of temperature, salinity, oxygen and present pCO_2 levels. For example, in the Baltic Sea pCO_2 concentrations up to 2,300 µatm have recently been measured in the Kiel Fjord[13], close to where the eastern Baltic cod stock spawns, and 1,200 µatm in the deep waters of the Bornholm Basin (Frommel *et al.*, manuscript in preparation), 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[14]. These high latitudes are assumed to be particularly impacted by future ocean acidification, owing to cold water, high primary productivity and melting of sea ice[15–17], and pH values are predicted to approach 7.7 over most of the coastal Arctic Ocean by 2100 (ref. 18). 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 CO_2 (ref. 19).

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 kidneys[20,21]. However, early life-history stages, lacking gills, may not be as competent in regulating their internal acid–base balance[6,7] and are thus predicted to be impacted more heavily by increasing pCO_2 levels.

In this study, we experimentally tested this prediction and exposed larvae of Norwegian coastal cod to three levels of pCO_2 (control: 380 µatm, medium: 1,800 µatm and high: 4,200 µatm) from newly fertilized eggs to seven weeks post-hatch (see Supplementary Information). Cod larvae are difficult to rear in the laboratory and require space, near-natural conditions and live prey to survive. Our large (2,300 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 allowed only three replicates. It also limited the ability to monitor larval numbers inside the tanks and, because 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 $\alpha = 5\%$) because of the small replicate number ($n = 3$) and substantial variation within treatments.

Larval growth was positively affected by high pCO_2 between 25 and 46 days post-hatch (dph) (Fig. 1). At 32 dph cod larvae from the high treatment had attained 59% more dry weight relative to the control whereas the medium treatment was not significantly different (Supplementary Table S1). 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

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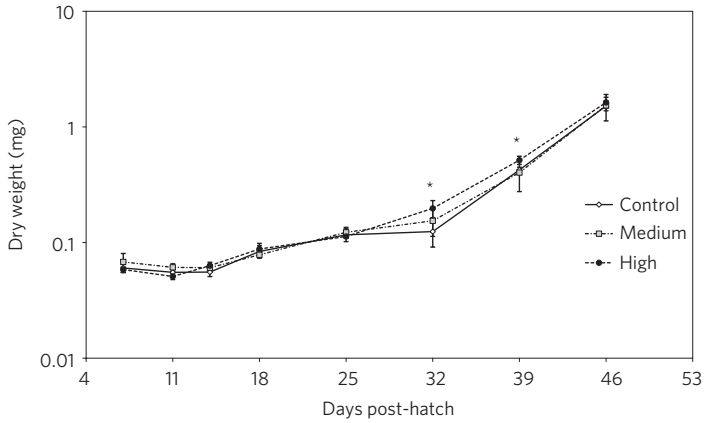

Figure 1 | Larval growth in dry weight over the entire experimental period. Dry weight with standard deviation for each of the three treatments over seven weeks post-hatch on a logarithmic scale. Asterisks indicate significant differences between control and high treatment. $N = 60$ larvae per replicate. For statistics see Supplementary Table S1.

with the developmental stages 8 and 9, a phase of intense transition where critical structural changes take place in all major landmarks[22,23]. 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 pCO_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[24] and may cause a shift from aerobic to anaerobic metabolism[25]. This in turn can influence protein biosynthesis[26]. Although 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 (Fig. 2). At 32 dph, cod larvae from the medium treatment had 61% increase and from the high treatment 97% increase in lipid content compared with the control (Supplementary Table S1). No significant difference in fatty acid composition could be identified (analysis of variance (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 pCO_2 treatments. Severe tissue damage was found in the liver, pancreas, kidney, eye and the gut of larvae 32 dph (Fig. 3), with the degree of damage significantly increasing with pCO_2 concentration (Fig. 4, Supplementary Table S2). Throughout the livers of CO_2 treated larvae, large lipid vacuoles were observed. Typical lipid vacuoles of control animals were in the order of 3–4 μm in diameter, whereas impacted cod had lipid vacuoles in the order of 7–9 μm in diameter. Furthermore, 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[27], which can lead to the observed necrosis.

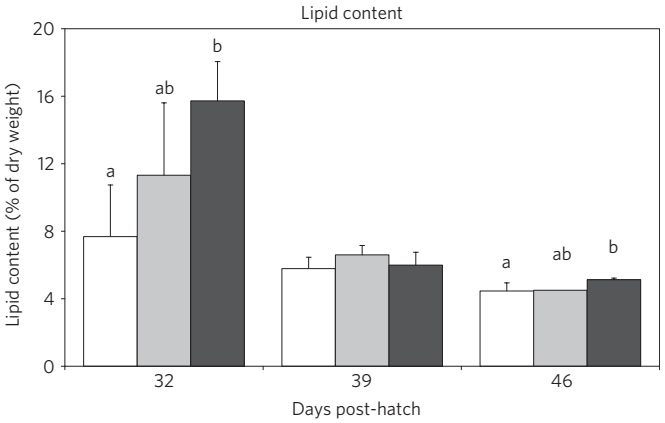

Figure 2 | Lipid content of larval cod for the last three sampling intervals. Mean lipid content of three replicates as a percentage of dry weight with standard deviation for each of the three treatments (control, white bars; medium, grey bars; high, black bars) at 32 dph, 39 dph and 46 dph. Letters indicate significant differences, 'a' is significantly different from 'b', but both are not significantly different from 'ab'. For test statistics, see Supplementary Table S1. $N = 30$ larvae per replicate.

Liver damage as a consequence of high pCO_2 concentrations has previously been found in freshwater fish[28], as well as in isolated Antarctic fish hepatocytes[26]. Similar to the liver, there was evidence of vacuolation in the epithelial cell cytoplasm of the kidney, with changes in the staining characteristics. Furthermore, 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 CO_2 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. Furthermore, the pigmented layer often had an irregular profile at increased pCO_2 concentrations. In the gut, the connective tissue was found to be highly fragile with the gut epithelium readily detaching from the basement membrane. Furthermore, bacteria were present in the gut

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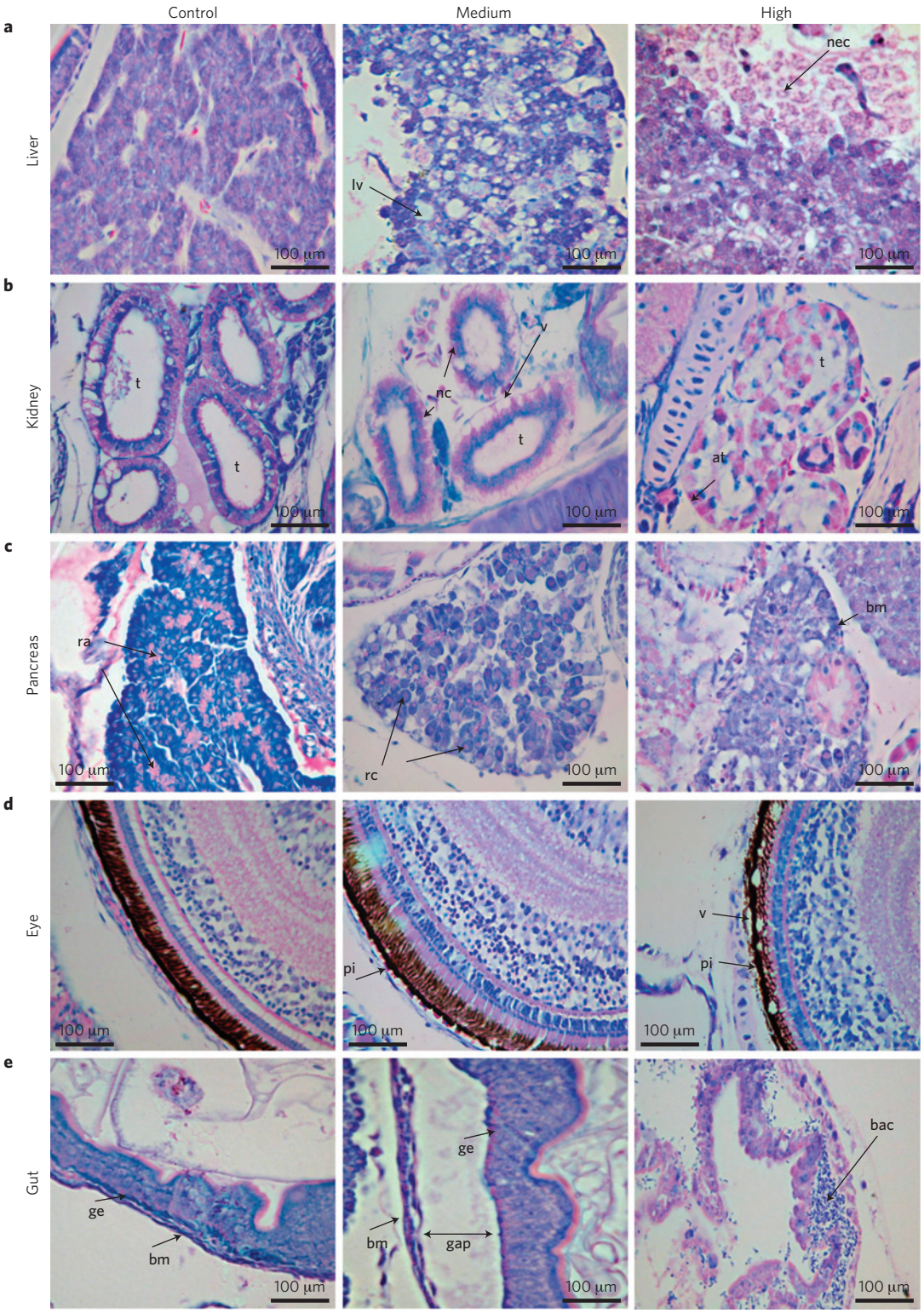

Figure 3 | Tissue damage from histological sections in larvae under increased pCO_2. Histological sections of liver (**a**), kidney (**b**), pancreas (**c**), eye (**d**) and gut (**e**) from cod larvae at three different pCO_2 treatments: control (left panels), medium (centre panels) and high (right panels). Note, gut sections are for control (left) and high (centre) treatments only as the connectivity issue of the gut was only found in the high treatment; bacteria (right) were found in the medium and high treatments. The structures identified in these sections are: liver: enlarged lipid vacuoles (lv), necrotic hepatocytes (nec); kidney: pronephric tubules (t), neck cells breaking up (nc); vacuoles (v), atrophy (at); 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).

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,

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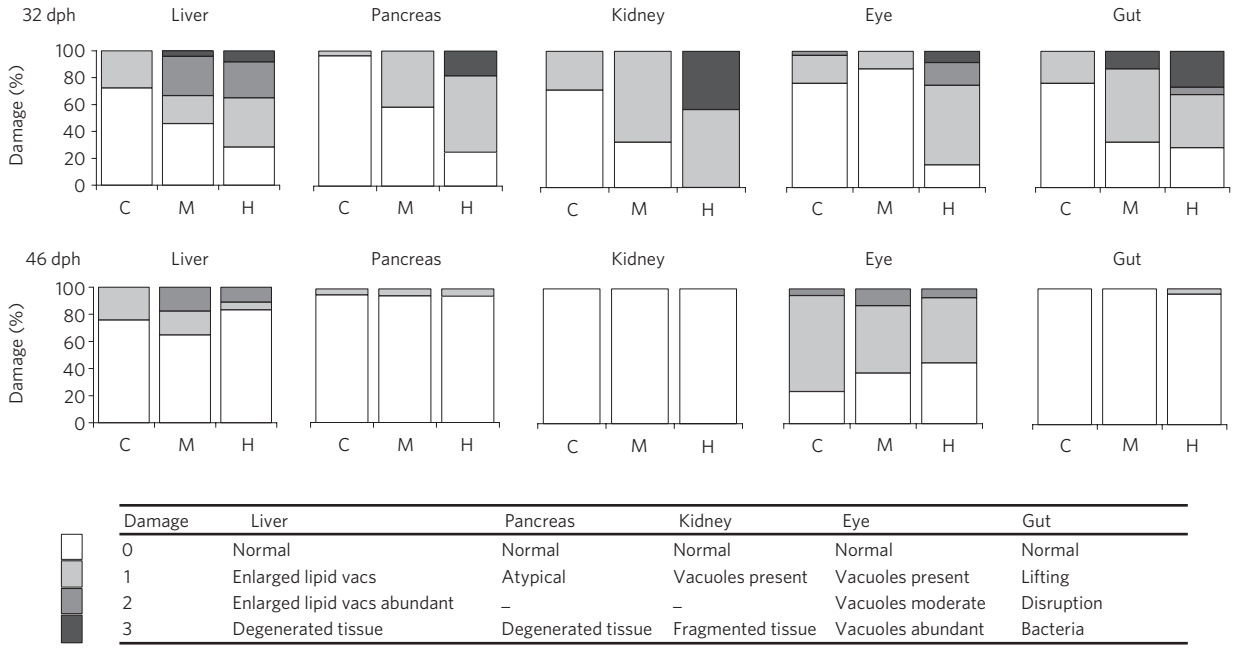

Damage	Liver	Pancreas	Kidney	Eye	Gut
0	Normal	Normal	Normal	Normal	Normal
1	Enlarged lipid vacs	Atypical	Vacuoles present	Vacuoles present	Lifting
2	Enlarged lipid vacs abundant	–	–	Vacuoles moderate	Disruption
3	Degenerated tissue	Degenerated tissue	Fragmented tissue	Vacuoles abundant	Bacteria

Figure 4 | Quantification of degree of damage in various organs with increasing pCO_2. Mean percentage of larvae at 32 and 46 dph showing the degree of damage (white bars are normal, with shading increasing with increasing severity of damage) at three different treatment levels (control (C), medium (M) and high (H)) for each different type of tissue. 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 S2 and S3.

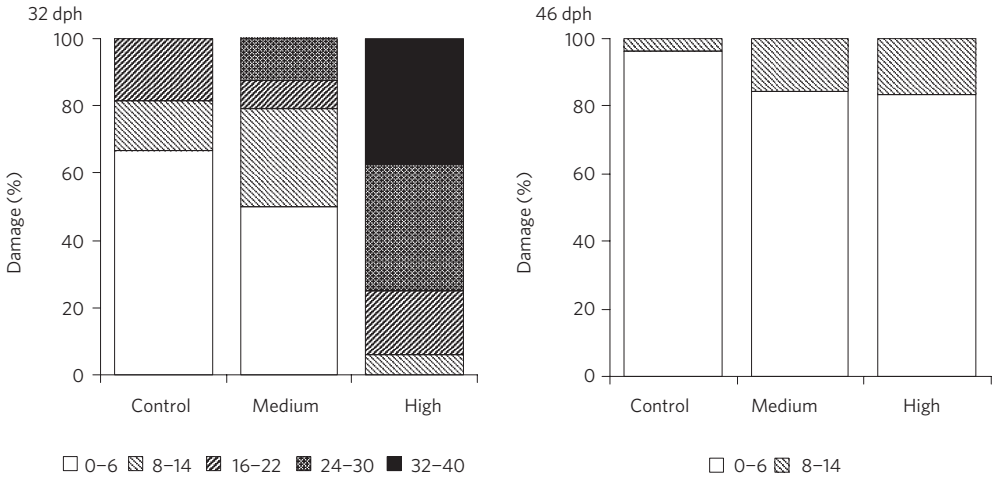

Figure 5 | Quantification of total damage to larvae at increasing pCO_2. Percentage of larvae exhibiting different degrees of total damage (calculated by the total index *Tot-I*) at 32 and 46 dph and three different treatment levels (control, medium and high). The damage is shown as five levels with normal as white bars, and shading increasing with increasing severity of damage.

this parameter was considered unsafe. No effects were found in the heart, gills, skeleton or skin.

Most of the histological damage found was 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 ref. 29). The health status of each larvae examined, calculated by the total index *Tot-I*, revealed that 12% of the larvae in the medium pCO_2 treatment and 75% of the larvae in the high pCO_2 treatment had severe damage in multiple tissues (Fig. 5). After the onset of gill-mediated acid–base regulation, this damage disappeared and no effect of CO_2 was found at day 46 (for statistics see Supplementary Table S3). This study demonstrates widespread tissue damage as a result of ocean acidification during a critical life-cycle phase within a mass-spawning, commercially important fish. Our data complement other studies that found behavioural effects in response to increased pCO_2 in tropical fish, which have a very different developmental pattern and a much faster developmental rate[8,9,30]. Laboratory experiments are always limited as they simulate increasing CO_2 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 ocean acidification is probably relatively slow on absolute time scales. Furthermore, like many other commercially exploited fish, cod already experience high selection pressure from 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 ocean acidification will negatively impact the recruitment of mass-spawning fishes because of enhanced mortality

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rates. Adverse effects of ocean acidification have thus to be added to the growing list of anthropogenic disturbances affecting already exploited fish stocks.

Received 16 May 2011; accepted 9 November 2011; published online 11 December 2011

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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, Espegrend, in cooperation with the University of Bergen. The authors are grateful to R. Bellerby and his lab for assistance with the carbonate chemistry and to H. Otteraa, V. Lokøy, F. Midtøy and C. 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.B.H.R. analysed data; A.Y.F. and T.B.H.R. wrote the main paper; All authors discussed the results and implications and commented on the manuscript at all stages.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper on www.nature.com/natureclimatechange. Reprints and permissions information is available online at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to A.Y.F.

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