Marine Pollution Bulletin 86 (2014) 154–160

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

The proteome of Atlantic herring (*Clupea harengus* L.) larvae is resistant to elevated pCO_2

Rommel H. Maneja [a,d], R. Dineshram [b], Vengatesen Thiyagarajan [b,*], Anne Berit Skiftesvik [c], Andrea Y. Frommel [d], Catriona Clemmesen [d], Audrey J. Geffen [e], Howard I. Browman [c,*]

[a] Center for Environment and Water, Research Institute, King Fahd University of Petroleum and Minerals, 31261 Dhahran, Saudi Arabia
[b] The Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong Special Administrative Region
[c] Institute of Marine Research, Austevoll Research Station, 5392 Storebø, Norway
[d] Helmholtz-Zentrum für Ozeanforschung Kiel-GEOMAR, Düsternbrooker Weg 20, 24105 Kiel, Germany
[e] Department of Biology, University of Bergen, PO Box 7803, N-5020 Bergen, Norway

ARTICLE INFO

Article history:
Available online 7 August 2014

Keywords:
Atlantic herring
Ocean acidification
Clupea harengus
Fish larvae
Environmental proteomics

ABSTRACT

Elevated anthropogenic pCO_2 can delay growth and impair otolith structure and function in the larvae of some fishes. These effects may concurrently alter the larva's proteome expression pattern. To test this hypothesis, Atlantic herring larvae were exposed to ambient (370 μatm) and elevated (1800 μatm) pCO_2 for one-month. The proteome structure of the larvae was examined using a 2-DE and mass spectrometry. The length of herring larvae was marginally less in the elevated pCO_2 treatment compared to the control. The proteome structure was also different between the control and treatment, but only slightly: the expression of a small number of proteins was altered by a factor of less than 2-fold at elevated pCO_2. This comparative proteome analysis suggests that the proteome of herring larvae is resilient to elevated pCO_2. These observations suggest that herring larvae can cope with levels of CO_2 projected for near future without significant proteome-wide changes.

1. Introduction

The unabated anthropogenic emission of CO_2 due to burning of fossil fuels has resulted in a phenomenon known as ocean acidification (OA). OA is characterized by an increasing concentration of CO_2 or partial pressure of CO_2 (pCO_2) and decreasing pH, carbonate ion concentration and saturation states of $CaCO_3$ minerals such as aragonite and calcite in the ocean (Feely et al., 2009). The early life stages of fish are particularly sensitive to environmental perturbations (Fuiman and Werner, 2009), including OA (e.g. Munday et al., 2010). The effect of OA on the larvae of fishes appears to be species and population specific and also depends upon the end-point measured. For example, the swimming kinematics and occurrence of feeding-related S-postures in Atlantic herring (*Clupea harengus*) larvae were robust to elevated levels of pCO_2 at 4200 μatm (Maneja et al. submitted). The swimming kinematics of Atlantic cod larvae (*Gadus morhua*) was also unaffected by 4200 μatm of pCO_2 (Maneja et al., 2013). Negative effects of high pCO_2 on larval growth and survival have been reported in the larvae of some species of marine fish (e.g. Frommel et al., 2012; Baumann et al., 2012), but not others (e.g. Hurst et al., 2012, 2013; Munday et al., 2011). Larval reef fish exposed to elevated pCO_2 (1000 to 1700 μatm) lose their ability to discriminate between ecologically important chemical cues, such as odors from different habitat types, kin and non-kin, and the smell of predators and prey (Dixson et al., 2010; Cripps et al., 2011; Munday et al., 2009). The response of fishes to auditory and visual cues also appears to be affected (Simpson et al. 2011; Ching et al., 2014) by elevated pCO_2 and a range of other behavioral problems have been detected, including the loss of behavioral lateralization and increased 'anxiety' (Domenici et al., 2012; Hamilton et al., 2014).

Some effects of elevated pCO_2 on marine teleosts, such as the behavioral responses referred to above, will be sub-lethal. Thus, further studies are required to understand the physiological processes and molecular mechanisms that are affected by elevated pCO_2 (e.g. Ishimatsu et al., 2005; Pörtner et al., 2005). Global protein expression patterns that characterize phenotypic variability and response plasticity (see Tomanek, 2011) can help us achieve that objective.

Expression proteomics, which assesses the global change in protein expression (Görg et al., 2004), is a useful tool in analyzing the environment's sublethal influence on organisms and their

* Corresponding authors.
E-mail addresses: rajan@hku.hk (V. Thiyagarajan), howard.browman@imr.no (H.I. Browman).

http://dx.doi.org/10.1016/j.marpolbul.2014.07.030
0025-326X/

adaptation to the environment (Diz et al., 2012). The proteome, which is the expressed protein complement of the genome, varies among tissues and over time, but represents the final and stable product of many redundant gene expression processes, making the final protein level a close approximation of the response of the organism (Campos et al., 2012; Lacerda and Reardon, 2009; López-Barea and Gómez-Ariza, 2006). For non-model organisms, the traditional 2-DE proteomic approach is suitable because differentially expressed proteins in a proteome can be displayed between different treatment levels (e.g. Dineshram et al., 2013; Mukherjee et al., 2013; Thiyagarajan et al., 2009). Although the 2-DE based proteome approach has been applied successfully to understand the effects of pollutants on the proteomes of several non-model species (Campos et al., 2012; Carpentier et al., 2008; McDonagh and Sheehan, 2006), its application in OA research is still in its infancy (Jones et al., 2013; Mykles et al., 2010; Tomanek, 2011). In this study, we analyzed the global proteomic expression of Atlantic herring (*C. harengus*) larvae cultured under ambient pCO_2 (370 μatm) and medium/elevated pCO_2 (1800 μatm) using a 2-DE based proteomic approach.

2. Materials and methods

2.1. Experimental animal and fish 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. The plates with fertilized eggs were distributed in such a way that one male-female cross was represented in all treatment tanks. The eggs remained under normal pCO_2 conditions for four days, after which pCO_2 levels were adjusted to the desired treatment level. Before the start of hatching, the plates were placed inside floating incubation buckets in each respective tank in order to monitor the hatching process and allow for the counting of the larvae. Fifty percent hatching occurred on April 16, 2010, which is designated as 0-day post hatch (DPH). During the 0-DPH, newly-hatched larvae were redistributed among the replicate tanks within each treatment to achieve equal initial stocking density of 4 larvae L^{-1} per tank. A 24-h filtration of the adjacent seawater using the Hydrotec size-selective filter system ensured a regular (daily) fresh supply of natural zooplankton with feeding density of 2000 prey L^{-1} (Seljeset et al., 2010). An initial prey size fraction of 80–250 μm was given and later increased to 350–500 μm.

To examine the effects of elevated pCO_2 on herring larval growth, larvae were sampled at eight different ages during the experiment (Fig. 1). Larvae were sampled by dropping a PVC-pipe with a manual closing mechanism into the water column. When the larvae reached a size at which they could escape from the pipe, they were collected with hand nets. Photographs of the fish larvae were taken and then the larvae were frozen at −80 °C. Standard length of each fish larva was later measured from calibrated photographs.

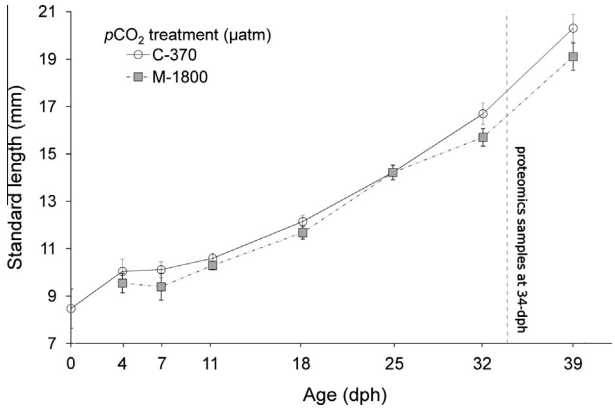

Fig. 1. Length of Atlantic herring (*Clupea harengus* L.) larvae cultured under two pCO_2 levels, control (C) −370 μatm and Medium (M) −1800 μatm for proteomics analysis at age 34 days post hatch.

This study was carried out in accordance with the laboratory regulations applicable in 2010, which are laid down in the Animal Welfare Act (LOV 2009-06-19 nr 97: Lov om dyrevelferd). Norway has entered into international agreements undertaken to follow EU Directive on laboratory animals (86/609/EEC) and the Council of Europe Convention on laboratory animals (ETS 123). The protocol was approved by the national regulatory Committee on the Ethics of Animal Experiments (Forsøksdyrutvalget) with Permit Number: ID2346. All conditions and sampling were conducted to minimize suffering.

2.2. Experimental design

The study was conducted in the land-based mesocosms at the University of Bergen's Espegrend Marine Station from March to May 2010. There were three pCO_2 treatment levels and three replicates per treatment. Carbon dioxide was introduced into the bottom of each 2650-L experimental tank by computer-controlled bubbling of CO_2 to achieve pCO_2 levels of 370 μatm (ambient or control), 1800 μatm (medium pCO_2) and 4200 μatm (high). Samples from the high pCO_2 treatment were not sufficient for proteomics analysis, therefore, only the ambient and medium treatments are reported upon.

Weekly seawater sampling was conducted for total alkalinity (TA) and dissolved inorganic carbon (DIC), which were used for the calculation of the total carbonate chemistry. To monitor proper functioning of the whole CO_2 delivery system, daily monitoring of pH levels using seawater buffer calibrated-WTW pH probe and checked with the seawater certified reference materials (supplied by Andrew Dickson, Scripps Institution of Oceanography) was also carried out. Further details on the seawater carbonate chemistry data observed in this study are provided in previous papers (Frommel et al., 2012; Maneja et al., 2013). The 2650-L experimental tanks were placed inside two water baths to buffer fluctuations in air temperature. Thus, a stable rise of temperature from 5 °C in March to 10 °C in May was achieved. This closely followed the temperature of seawater at 40-m depth intake near the marine station. Seawater in the mesocosms had a stable salinity of 33.3 psu and dissolved oxygen concentration above 90% saturation. Supply of seawater to the experimental and buffer tanks was done using a flow-through system.

The herring larvae used in this proteomics study were obtained from live larvae used for swimming behavior observations (Maneja et al. submitted). They were kept under the same water conditions as in the main mesocosm tanks throughout the video recording of the swimming behavior (see Maneja et al., 2013). The 34 DPH larvae were sampled from the upper water column of each of the experimental tank. After each replicate had been video recorded, the herring larvae were transferred to Eppendorf vials and frozen immediately at −80 °C. Larval lengths for the herring larvae at 34 DPH were estimated from the growth rates of the larvae between 32 and 39 DPH.

2.3. Sample preparation for two-dimensional electrophoresis (2-DE)

Plankton and other particles adhering to the frozen larvae were cleaned and removed through forcefully washing with Milli-Q water. The larvae were then rapidly frozen using dry ice or liquid nitrogen and lyzed in 2-DE buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM dithiothreitol and 2% Bio-Lyte 3/10 ampholyte) (Görg et al., 2004; Thiyagarajan and Qian, 2008) using a sonicator

(Branson Sonifier 150) with setting 3 (6 rounds of 35 s with two min pause-interval). Larval samples were stored on ice during sonication to avoid the denaturing of proteins. The solubilized larval proteins (in 2-DE buffer) were centrifuged for 20 min at 1400 rpm. A modified Bradford method was used to quantify the soluble proteins in the supernatant (Ramagli and Rodriguez, 1985), which was then used for 2-DE analysis.

2.4. 2-DE analysis

The optimized larval proteomic protocols adapted from several previous studies were followed to separate the larval proteins in a 2-DE analysis (Thiyagarajan and Qian, 2008; Thiyagarajan et al., 2009). The proteomes were obtained by separating 400 μg of larval proteins in two dimensions (horizontal: separated using isoelectric focusing strip pI range 3–10 linear, and vertical: on gel electrophoresis with mass 14–64 kDa). The separated proteins were then visualized using quantitative silver staining method. A total of six 2-DE gels were analyzed (2 treatments × 3 replicates). The gels were scanned using BIO-RAD GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) at an optical resolution of 300 dpi. The number and expression of proteins in the gels were then analyzed using Progenesis Samespots (ver. 4.5; Nonlinear Dynamics). Abundantly and differentially expressed proteins between pCO_2 treatments were selected for subsequent identification based on the following criteria: (1) ≥2-fold difference in spot abundance, and (2) consistent change among the replicates ($p < 0.05$, one way ANOVA test) (Carpentier et al., 2008).

2.5. Protein identification using MALDI–TOF/TOF mass spectrophotometer

Twelve differentially expressed protein spots were excised for mass spectrometric identification according to the standard protocol (Shevchenko et al., 1996). The gel pieces (protein spots) were washed twice for 15 min each with water and twice with water/acetonitrile (1:1 v/v) and were then placed in 100% acetonitrile. Protein spots were dried in a Speed Vac centrifuge before adding 10 μl of 20 ng/μl sequencing grade trypsin (Promega) in 20 mM NH_4HCO_3 buffer. After rehydration with the enzyme solution, the gel pieces were covered with the buffer solution and digestion was allowed to proceed overnight at 37 °C. The peptides were extracted using several volumes of an water/acetonitrile/trifluoroacetic acid mixture (80:20:1), after clean up with ZipTip (Millipore), peptides subjected to mass/charge ratio analysis using MALDI–TOF/TOF mass spectrometer (ABI 4800 Proteomics Analyzer, Applied Biosystems). Combined peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS) information obtained from the MS process was subjected to searching against the National Center for Biotechnology Information (NCBI) and Expressed Sequence Tags (EST) invertebrate database with entities restricted to other-metazoan sequences using the GPS Explorer algorithm, ver. 3.6 (Applied Biosystems) and in-house MASCOT database ver. 2.2 (Matrix Science). Mass spectrum searches were performed using mass tolerance settings of ±75 ppm for PMF and ±0.2 Da for the MS/MS spectra. MASCOT scores greater than 69 ($p < 0.05$) and ion score of minimum one peptide greater than 50 ($p < 0.05$) or three peptides greater than 20 ($p < 0.05$) were considered significant (Shevchenko et al., 2001).

3. Results

3.1. Effect of elevated pCO_2 on larval growth

A significant decrease in the standard length of Atlantic herring larvae with increase in pCO_2 was observed except at 0 and 7 DPH (Nested-ANOVA, $p < 0.01$) (Fig. 1). The specific growth rates estimated using daily increase in length between 32 and 39 DPH were 0.52 mm d^{-1} (ambient pCO_2) and 0.49 mm d^{-1} (medium pCO_2 level: hereafter referred to as "elevated pCO_2"). Standard length of herring larvae at 32 DPH ranged from 14.57 to 19.44 mm in the control group and 11.23–19.48 mm in the medium treatment. At 39 DPH, sizes ranged from 15.94 to 23.18 mm in the control group and 15.92–20.78 mm in the medium treatment. Based upon an interpolation of the growth rates between 32 and 39 DPH, the mean lengths of the 34 DPH larvae used for the proteomics analysis were 17.7 mm for ambient and 16.7 mm for the elevated pCO_2 (medium) treatments.

3.2. Protein expression in larval Atlantic herring under elevated pCO_2

The 2-DE protocol used in this study identified over 649 proteins from the 2-DE lysis buffer soluble proteins. The quality and the number of protein spots visualized on the 2-DE gels of the herring larvae samples were distinct and the edges of the spots were clear (Fig. 2). The majority of proteins in the larvae were visualized in the acidic region of the gel (pI 3 to 7) and were of low-molecular weight, ranging between 40 and 10 kDa. A representative 2-DE proteomic map of herring larvae from the ambient (control) and elevated (medium) pCO_2 groups is shown in Fig. 3. By visual inspection, the overall protein expression pattern did not show any obvious up or down-regulation of proteins in the elevated pCO_2 treatment. This similarity between the groups was evident from all of the three biological replicated gel images (Fig. 3). This visual image analysis is corroborated by the results of proteome image analysis. When a 2-fold or higher difference in spot intensity was considered significant ($p < 0.05$), there was no difference between the control and elevated pCO_2 groups (Students t test, $p > 0.05$).

When a less stringent threshold criterion for spot intensity (i.e. 1.5-fold) was applied to assess the possible effect of pCO_2 on the proteome of herring larvae, there was a difference between the control and treatment groups (Students t test, $p < 0.05$). However, the difference between the groups was due to only 10 down-regulated and 9 up-regulated proteins. A volcano plot analysis illustrated the difference in protein expression levels between control and elevated pCO_2 groups considering a p-value cutoff of 0.05 and a threshold fold-change cutoff of 1.5 (up- or down-regulated) (Fig. 4). Subsequently, a principal component analysis (PCA) of the

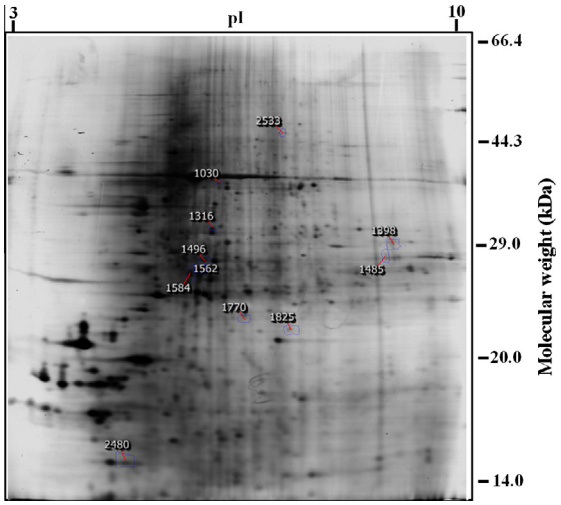

Fig. 2. A typical proteome map of the Atlantic herring (*Clupea harengus* L.) larvae obtained from standard 2-DE analysis. The marked protein spots were identified using MALDI-TOF mass spectrophotometry.

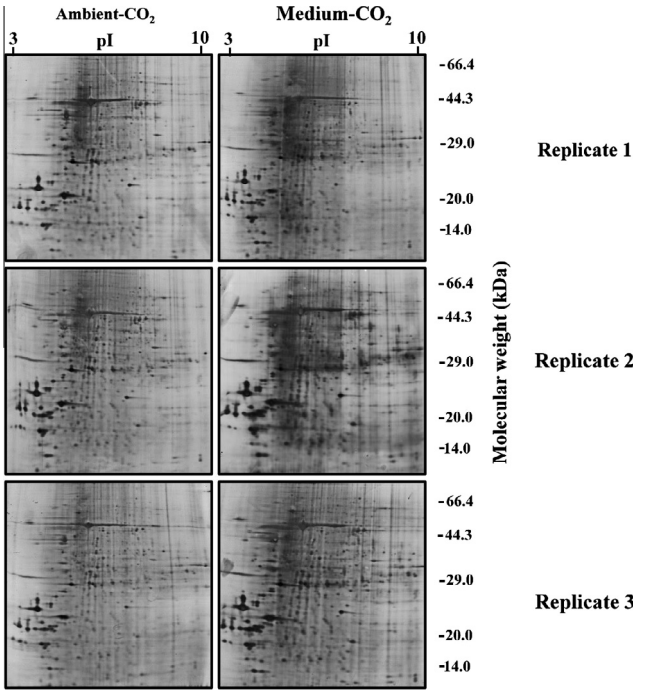

Fig. 3. Effects of elevated pCO_2 (Medium pCO_2 −1800 μatm) on Atlantic herring (*Clupea harengus* L.) larval protein expression on a 2-DE map was shown using three biologically independent samples.

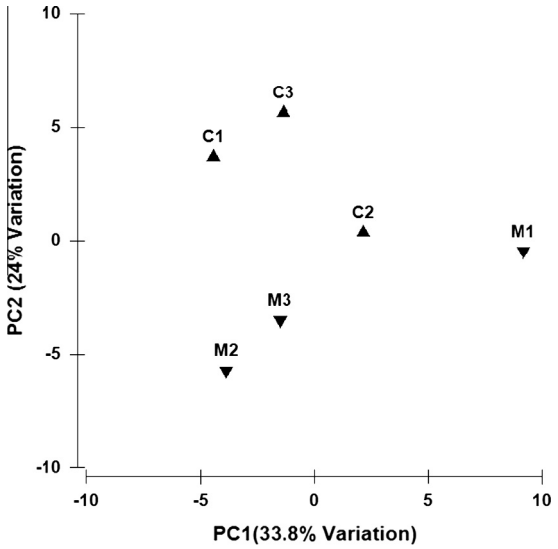

Fig. 5. Principal Component Analysis (PCA) showing the effect of elevated pCO_2 on Atlantic herring (*Clupea harengus* L.) larval proteome structure. Euclidian distances summed over spots and Ward's method for all protein spots in gels was used for PCA analysis. C: ambient or control pCO_2 level of 370 μatm; M: elevated pCO_2 level of 1800 μatm.

proteome also differentiated control samples from the elevated pCO_2 group (Fig. 5). Principal component 1 (pI and MW) explained 33.8% of the variation. Similarities in the global expression pattern of replicate gels in both groups were estimated using a TMEV heat map that resulted from hierarchical clustering analysis of normalized protein spot volume data of all six samples. The proteome of samples were clearly grouped in two well-differentiated clusters, the control and the elevated pCO_2 treatment (Fig. 6).

3.3. Identification of differentially expressed proteins in under elevated pCO_2 conditions

All of the 19 differentially expressed proteins between the groups were subjected to MALDI-TOF/TOF analyses using the

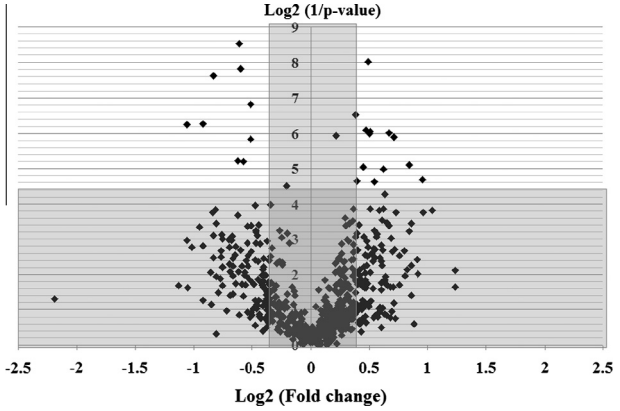

Fig. 4. Volcano plot of Atlantic herring (*Clupea harengus* L.) larval proteome data showing the effect of elevated pCO_2 (i.e. Medium −1800 μatm) on the expression pattern of proteins when compared to ambient control level of 370 μatm. The protein spot intensity data from replicate proteome gel images was obtained using Progenesis SameSpots analysis software. The up- or down-regulated proteins are those that are lying outside the shaded area in the plot.

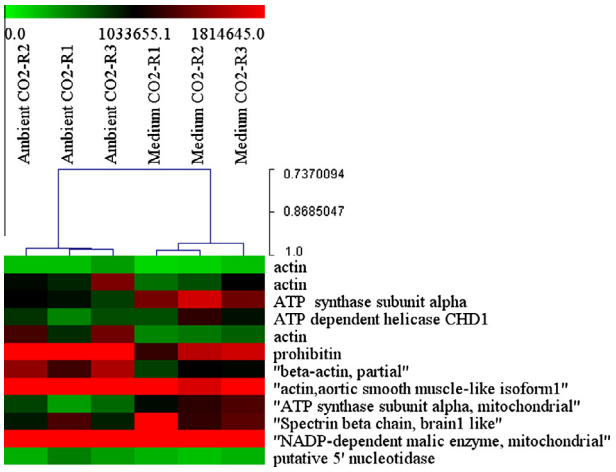

Fig. 6. Heat map of differentially expressed proteins of the Atlantic herring (*Clupea harengus* L.) larvae in response to elevated pCO_2. The protein intensity in the pCO_2 treatment and control groups are illustrated in different color codes. The hierarchy analysis shown along with the heat map show the similarity among replicate samples within a control or treatment group as well as difference between control and pCO_2 treatment.

combined PMF MS/MS strategy for the identification. However, only 12 of them were identified with high confidence. The remaining seven proteins could not be identified owing to the lack of sequenced genomic information for Atlantic herring. The identified proteins are listed in Table 1 and marked on the gel image in Fig. 2. There was a slight discrepancy between the theoretical MW and pI and those obtained experimentally from the gel. The PMF and MS/MS results suggested that this could be due to the low sequence coverage of the analyzed peptide fragments in the database, which is common in non-model species (Kultz et al., 2007). Seven of these were down-regulated while five of them were up-regulated (Table 1). Most of the up-regulated protein spots were identified as muscle proteins while down-regulated proteins were involved in metabolism.

Table 1
List of identified and differentially expressed protein of the Atlantic herring larvae (*Clupea harengus* L.) in response to elevated pCO_2.

Spot[a]	Putative identification[b]	Total protein score[c]	Peptide count[d]	Homology to protein (NCBI accession no. and species name)[e]	Fold change	*p*-value
1316	Actin	399 (100)*	13	gi|37903435 *Danio rerio*	−1.4	0.0008
1030	Actin	492 (100)	16	gi|37903435 *Danio rerio*	−1.5	0.002
1398	ATP synthase subunit alpha	113(99.9)*	4	gi|40386586 *Amphimedon queenslandica*	1.5	0.015
1485	ATP dependent helicase CHD1	65*	17	gi|269969347 *Gallus gallus*	1.4	0.003
1496	Actin	321 (100)*	13	gi|345322034 *Ornithorhynchus anatinus*	−1.9	0.013
1562	Prohibitin	371 (100)*	9	gi|213515458 *Salmo salar*	−1.8	0.013
1584	Beta-actin, partial	192 (100)	5	gi|374413852 Sardina pilchardus	−1.5	0.004
1770	ATP synthase subunit alpha, mitochondrial	101 (99.9)*	12	gi|116325975 *Danio rerio*	1.9	0.038
1825	Spectrin beta chain, brain1 like	65*	31	gi|345481110 *Nasonia vitripennis*	1.4	0.014
2480	NADP-dependent malic enzyme, mitochondrial	62*	10	gi|346716344 *Sus scrofa*	1.7	0.029
2533	Putative 5′ nucleotidase	66*	8	gi|190702164 *Glyptapanteles flavicoxis*	−1.4	0.017
1611	Actin, aortic smooth muscle-like isoform1	549 (100)*	16	gi|297301436 *Macaca mulatta*	−1.8	0.005

[a] The assigned spot number.
[b] Name of the putatively identified protein.
[c] The in-house MASCOT Total protein score obtained through searching against the Herrings NCBI and Metazoan NCBI database (MASCOT score >69 is considered significant).
[d] Number of matched peptides.
[e] Genbank accession number.
* The protein is identified by the Metazoan NCBI database.

4. Discussion

4.1. Herring larval growth at elevated pCO_2

Herring larvae from the elevated pCO_2 treatment had reduced somatic growth relative to the larvae from the control group (Fig. 1). The reduction in fish size with pCO_2 was also reflected in the developmental state of the larvae, with the control group further developed than the medium group at 39 DPH (see Fig. 3 in Frommel et al., 2014). The reduction in somatic growth might be indicative of sub-lethal physiological effects of elevated pCO_2 on herring larvae such as, for example, the additional metabolic cost of maintaining internal homeostasis (e.g. Fabry et al., 2008; Pörtner et al. 2004). Expensive metabolic activities such as growth and reproduction can be suppressed when the required protein synthesis is reduced (Fabry et al., 2008; Kroeker et al., 2010). Reduction in protein synthesis was inferred from a significant decrease of RNA/DNA ratio observed in 39 DPH herring larvae from the medium treatment relative to the control (Frommel et al., 2014). Previously, we reported that elevated pCO_2 only had negative effects on the RNA/DNA ratio of newly hatched herring larvae but did not affect larval length, dry weight, yolk sac area or otolith area (Franke and Clemmesen, 2011). It was proposed that the reduction in RNA/DNA ratio with pCO_2 would lead to reduced somatic growth with age. The results presented here, and in a previous study (Frommel et al., 2013) support this conclusion. It should be noted, however, that reduction in RNA/DNA ratio was not always associated with a reduction in fish size (Franke and Clemmesen, 2011). Reduction in growth and delayed development caused by elevated pCO_2 has also been reported in other marine organisms, e.g. larval sea urchin (Stumpp et al., 2011) and larval Pacific oysters (Dineshram et al., 2012; Timmins-Schiffman et al., 2013).

4.2. Herring larval 2D-protein profile

649 protein spots were identified from Atlantic herring larvae. The number of protein spots expressed in the herring larvae from the control group was comparable to that for the proteomes of other fish larvae: 422 spots in 6 DPH Atlantic cod larvae (Sveinsdóttir and Gudmundsdóttir, 2010), 374 and 428 spots in 6 and 24 DPH Atlantic cod larvae, respectively (Sveinsdóttir et al., 2008, 2009), 450 spots in 6 DPH Atlantic cod larvae (Sveinsdóttir and Gudmundsdottir, 2011).

4.3. The herring proteome under elevated pCO_2 conditions

When a threshold level of >2-fold was applied, there was no difference in the global protein expression pattern between the control and elevated pCO_2 larvae, suggesting that herring larvae possess adequate acclimation mechanisms to tolerate near-future levels of pCO_2, at least with respect to their production of protein. The proteome of a marine coccolithophore (*Emiliania huxleyi*) was also resistant to elevated pCO_2 (Jones et al., 2013). The absence of a strong difference in the proteome of the two treatment groups also indicates that there was no major effect of between-treatment differences in size or developmental state.

Although no treatment-related difference in the proteome of herring larvae was identified using a >2-fold threshold, a lower threshold cut off value of 1.5 was also applied in order to assess the possibility that low abundance proteins were differentially expressed between treatments. At the lower threshold, 19 proteins were differentially expressed in the elevated pCO_2 treatment relative to the control. These differentially expressed proteins were tentatively identified as being pCO_2-sensitive (Table 1). This is consistent with an increasing number of reports highlighting proteome-level changes in the response of marine larval forms to elevated pCO_2. For example, a down-regulation of about 50% of the proteins mostly with low molecular weights (<40 kDa), which could be associated with larval metabolic depression, were reported in Pacific oyster larvae grown under 2275 µatm pCO_2 (pH 7.5) from embryo until 6 days post fertilization (Dineshram et al., 2012).

Previous studies on the global proteome of fish larvae reported >3-fold changes in abundance of 13 proteins involved in energy metabolism and development in Atlantic cod larvae (*G. morhua*) larvae (at 6 DPH) fed with either normal or protein hydrolysate-enriched food (Sveinsdóttir and Gudmundsdottir, 2010). The observed differences in protein expression were attributed to the enriched diet. Up-regulation (>2-fold change) of 3 (out of 269) and 2 (out of 169) protein spots were reported from gill tissue of Atlantic cod (*G. morhua*) and stickleback (*Gasterosteus aculeatus*), respectively, which were cultured at pH 7.7 for three weeks (Gunnarsson, 2010). Atlantic cod exposed to elevated pCO_2 levels (0.3 and 0.6 kPa) for 12 months exhibited a 2-fold increase in the

expression of ATPase, which corresponded to 2-fold increase in Na^+/K^+-ATPase activity (Melzner et al., 2009). Unlike the fish larval proteome response, about 12% of proteins in the mantle tissues of an adult oyster (*Crassostrea virginica*) changed expression under elevated pCO_2 conditions (Tomanek et al., 2011). Interestingly, cytoskeleton-related proteins were up-regulated in both the mantle tissue of oysters (Tomanek et al., 2011) and in the fish larval proteome (this study).

Interpretation of changes of the global proteome associated with exposure to elevated pCO_2 must consider the possibility of a confounding differential protein expression due to between-treatment differences in the size and developmental state of the larvae. The mean size of herring larvae differed by an average of only 1 mm in the treatment relative to the control group, and the size differences between a large percentage of the individuals was much less than 1 mm. Further, the larvae in the elevated pCO_2 treatment group were generally less developed than those in the control group, >50% of larvae in both treatment groups were at stage 3a (see Fig. 3 in Frommel et al., 2014). The slight changes (at less than 1.5-fold threshold change) in the 19 out of the 649 proteins could be due to this minor delay in growth and development. We would expect protein changes due to elevated pCO_2 to occur at much higher fold thresholds (e.g. Sveinsdóttir and Gudmundsdottir, 2010; Gunnarsson, 2010; Melzner et al., 2009).

In summary, the conventional but highly reproducible 2-DE technique was successfully employed in this study to assess the possible response of the Atlantic herring (*C. harengus*) larval proteome to present-day ambient and elevated pCO_2 (1800 µatm). When a stringent threshold level (>2-fold) was used to analyze proteome data, the larval proteome was interpreted to be resilient to elevated pCO_2. Even when the threshold level was lowered to 1.5, only 3% of the proteome was affected (19 of 649 proteins), further supporting the conclusion of resilience. Although 2-DE proteome map analysis is a powerful tool to examine overall proteome-level responses, the role of low abundance proteins, especially those associated with membranes, may have been overlooked in this study due to inherent limitations of the technique. Future studies should focus on low abundance proteins that are differentially expressed in response to elevated pCO_2 using quantitative proteomics approaches such as isobaric tags for relative and absolute quantification (iTRAQ).

Acknowledgements

The authors would like to thank the following colleagues who contributed significantly to the study: (1) Espegrend mesocosm experiment team- Dr. Mei-Yu Chang, Frank Midtøy, Vibeke Lokøy, Dr. Knut W. Vollset, Oddbjørn Seljeset, Agnes Aadnesen, Tomas Sørlie, Gladys Maneja, Håkon Otterå. (2) Institute of Marine Research Austevoll team- Dr. Yuichi Fukunishi. (3) Water chemistry team- Dr. Richard Bellerby, Hildegunn Almelid, Jane Strømstad. 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), by funds from the Fram - High North Research Centre for Climate and the Environment China-Norway Incentive Funds Project (to HIB), and by the Norwegian Institute of Marine Research ("Fine-scale 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.

References

Baumann, H., Talmage, S.C., Gobler, C.J., 2012. Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat. Climate Change 2, 38–41. http://dx.doi.org/10.1038/nclimate1291.

Cripps, I.L., Munday, P.L., McCormick, M.I., 2011. Ocean acidification affects prey detection by a predatory reef fish. PLoS ONE 6 (7), e22736. http://dx.doi.org/10.1371/journal.pone.0022736.

Campos, A., Tedesco, S., Vasconcelos, V., Cristobal, S., 2012. Proteomic research in bivalves: towards the identification of molecular markers of aquatic pollution. J. Proteomics 75, 4346–4359.

Carpentier, S.C., Panis, B., Vertommen, A., Swennen, R., Sergeant, K., Renaut, J., Laukens, K., Witters, E., Samyn, B., Devreese, B., 2008. Proteome analysis of non-model plants: a challenging but powerful approach. Mass Spectrom. Rev. 27, 354–377.

Ching, W.-S., Marshall, N.J., Watson, S.-A., Munday, P.L., Nilsson, G.E., 2014. Ocean acidification slows retinal function in a damselfish through interference with $GABA_A$ receptors. J. Exper. Biol. 217, 323–326.

Dineshram, R., Lane, A., Wang, K., Xiao, S., Yu, Z., Qian, P.Y., Thiyagarajan, V., 2012. Analysis of Pacific oyster larval proteome and its response to high-CO_2. Mar. Pollut. Bull. 64, 2160–2167.

Dineshram, R., Thiyagarajan, V., Lane, A., Ziniu, Y., Xiao, S., Leung, P.Y., 2013. Elevated CO_2 alters larval proteome and its phosphorylation status in the commercial oyster, *Crassostrea hongkongensis*. Mar. Biol. 160, 2189–2205.

Dixson, D.L., Munday, P.L., Jones, G.P., 2010. Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. Ecol. Lett. 13, 68–75.

Domenici, P., Allan, B., McCormick, M.I., Munday, P.L., 2012. Elevated carbon dioxide affects behavioral lateralization in a coral reef fish. Biol. Lett. 8 (1), 78–81.

Diz, A.P., Martinez-Fernandez, M., Rolan-Alvarez, E., 2012. Proteomics in evolutionary ecology: linking the genotype with the phenotype. Mol. Ecol. 21, 1060–1080.

Fabry, V.J., Seibel, B.A., Feely, R.A., Orr, J.C., 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J. Mar. Sci. 65, 414–432.

Feely, R.A., Orr, J., Fabry, V.J., Kleypas, J.A., Sabine, C.L., Langdon, C., 2009. Present and future changes in seawater chemistry due to ocean acidification. Geophys. Monogr. 183, 175–188.

Franke, A., Clemmesen, C., 2011. Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus* L.). Biogeosci. Discuss. 8, 7097–7126.

Frommel, A.Y., Maneja, R.H., Lowe, D., Malzahn, A.M., Geffen, A.J., Folkvord, A., Piatkowski, U., Reusch, T., Clemmesen, C., 2012. Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nat. Climate Change 2, 42–46. http://dx.doi.org/10.1038/nclimate1324.

Frommel, A., Schubert, A., Piatkowski, U., Clemmesen, C., 2013. Egg and early larval stages of Baltic cod, Gadus morhua, are robust to high levels of ocean acidification. Mar. Biol. 160, 1825–1834.

Frommel, A.Y., Maneja, R., Lowe, D., Pascoe, C.K., Geffen, A.J., Folkvord, A., Piatkowski, U., Clemmesen, C., 2014. Organ damage in Atlantic herring larvae as a result of ocean acidification. Ecol. Appl. 24, 1131–1143. http://dx.doi.org/10.1890/13-0297.1.

Fuiman, L.A., Werner, R.G., 2009. Fishery Science: the Unique Contributions of Early Life Stages. Wiley. com.

Görg, A., Weiss, W., Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics 4, 3665–3685.

Gunnarsson, F., 2010. Sublethal effects of low pH in two fish species (*Gasterosteus aculeatus* and *Gadus morhua*). M.Sc. Thesis, University of Gothenburg. 33pp.

Hamilton, T.J., Holcombe, A., Tresguerres, M. 2014 CO_2-induced ocean acidification increases anxiety in Rockfish via alteration of GABAA receptor functioning. In: Proc. R. Soc. B 281: 20132509. http://dx.doi.org/10.1098/rspb.2013.2509.

Hurst, T.P., Fernandez, E.R., Mathis, J.T., Miller, J.A., Stinson, C.M., Ahgeak, E.F., 2012. Resiliency of juvenile walleye pollock to projected levels of ocean acidification. Aquatic Biology 17, 247–259. http://dx.doi.org/10.3354/ab00483.

Hurst, T.P., Fernandez, E.R., Mathis, J.T., 2013. Effects of ocean acidification on hatch size and larval growth of walleye pollock (*Theragra chalcogramma*). ICES J. Mar. Sci. 70 (4), 812–822. http://dx.doi.org/10.1093/icesjms/fst053.

Ishimatsu, A., Hayashi, M., Lee, K.-S., Kikkawa, T., Kita, J., 2005. Physiological effects on fishes in a high-CO_2 world. J. Geophysical Res.: Oceans 110, C09S09.

Jones, B.M., Iglesias-Rodriguez, M.D., Skipp, P.J., Edwards, R.J., Greaves, M.J., Young, J.R., Elderfield, H., O'Connor, C.D., 2013. Responses of the *Emiliania huxleyi* proteome to ocean acidification 8, e61868.

Kultz, D., Fiol, D., Valkova, N., Gomez-Jimenez, S., Chan, S.Y., Lee, J., 2007. Functional genomics and proteomics of the cellular osmotic stress response in 'non-model' organisms. J. Exp. Biol. 210, 1593–1601.

Kroeker, K.J., Kordas, R.L., Crim, R.N., Singh, G.G., 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol. Lett. 13, 1419–1434.

Lacerda, C.M.R., Reardon, K.F., 2009. Environmental proteomics: applications of proteome profiling in environmental microbiology and biotechnology. Brief. Funct. Genomics Proteomics 8, 75–87.

López-Barea, J., Gómez-Ariza, J.L., 2006. Environmental proteomics and metallomics. Proteomics 6, 51–62.

Maneja, R.H., Frommel, A.Y., Browman, H.I., Clemmesen, C., Geffen, A.J., Folkvord, A., Piatkowski, U., Durif, C.M.F., Bjelland, R., Skiftesvik, A.B., 2013. The swimming

kinematics of larval Atlantic cod, *Gadus morhua* L., are resilient to elevated seawater pCO_2. Mar. Biol. 160, 1963–1972.
McDonagh, B., Sheehan, D., 2006. Redox proteomics in the blue mussel *Mytilus edulis*: carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. Aquat. Toxicol. 79, 325–333.
Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.O., 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6, 2313–2331.
Mukherjee, J., Wong, K., Chandramouli, K., Qian, P., Leung, P., Wu, R., Thiyagarajan, V., 2013. Proteomic response of the marine invertebrate larvae to acidification and hypoxia at the time of metamorphosis and calcification. J. Exp. Biol. (in press).
Munday, P.L., Dixson, D.L., Donelson, J.M., Jones, G.P., Pratchett, M.S., Devitsina, G.V., Døving, K.B., 2009. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc. Natl. Acad. Sci. 106, 1848–1852.
Munday, P.L., Dixson, D.L., McCormick, M.I., Meekan, M., Ferrari, M.C., Chivers, D.P., 2010. Replenishment of fish populations is threatened by ocean acidification. Proc. Natl. Acad. Sci. 107, 12930–12934.
Munday, P.L., Gagliano, M., Donelson, J.M., Dixson, D.L., Thorrold, S.R., 2011. Ocean acidification does not affect the early life history development of a tropical marine fish. Mar. Ecol. Prog. Ser. 423, 211–221.
Mykles, D.L., Ghalambor, C.K., Stillman, J.H., Tomanek, L., 2010. Grand challenges in comparative physiology: Integration across disciplines and across levels of biological organization. Integr. Comp. Biol. 50, 6–16.
Pörtner, H.O., Langenbuch, M., Reipschläger, A., 2004. Biological impact of elevated ocean CO_2 concentration: lessons from animal physiology and Earth history. J . Oceanogr. 60, 705–718.
Pörtner, H.O., Langenbuch, M., Michaelidis, B., 2005. Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: from earth history to global change. J. Geophysical Res. 110, C09S10.
Ramagli, L.S., Rodriguez, L.V., 1985. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis 6, 559–563.
Seljeset, O., Vollset, K.W., Folkvord, A., Geffen, A.J., 2010. The role of prey concentration and size range in the growth and survival of larval cod. Mar. Biol. Res. 6, 251–262.
Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.
Shevchenko, A., Sunyaev, S., Loboda, A., Shevchenko, A., Bork, P., Ens, W., Standing, K.G., 2001. Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. Anal. Chem. 73, 1917–1926.
Simpson, S.D., Munday, P.L., Wittenrich, M.L., Manassa, R., Dixson, D.L., Gagliano, M., Yan, H.Y., 2011. Ocean acidification erodes crucial auditory behavior in a marine fish. Biol. Lett. 7 (6), 917–920.
Stumpp, M., Dupont, S., Thorndyke, M., Melzner, F., 2011. CO_2 induced acidification impacts sea urchin larval development II: gene expression patterns in pluteus larvae. Comp. Biochem. Physiol. Part A 160, 320–330.
Sveinsdóttir, H., Gudmundsdóttir, A., 2010. Proteome profile comparison of two differently fed groups of Atlantic cod (*Gadus morhua*) larvae. Aquacult. Nutr. 16, 662–670.
Sveinsdóttir, H., Gudmundsdottir, A., 2011. Proteome analysis of abundant proteins in early atlantic cod (*Gadus morhua*) larvae. J. Icelandic Agric. Sci. 24, 23–31.
Sveinsdóttir, H., Vilhelmsson, O., Gudmundsdóttir, Á., 2008. Proteome analysis of abundant proteins in two age groups of early Atlantic cod (*Gadus morhua*) larvae. Comp. Biochem. Physiol. Part D 3, 243–250.
Sveinsdóttir, H., Steinarsson, A., Gudmundsdóttir, Á., 2009. Differential protein expression in early Atlantic cod larvae (*Gadus morhua*) in response to treatment with probiotic bacteria. Comp. Biochem. Physiol. Part D 4, 249–254.
Thiyagarajan, V., Qian, P.Y., 2008. Proteomic analysis of larvae during development, settlement, and metamorohosis in the fouling barnacle, *Balanus amphitrite*. Proteomics 8, 3164–3172.
Thiyagarajan, V., Wong, T., Qian, P.Y., 2009. 2D Gel-based proteome and phosphoproteome analysis during larval metamorphosis in two major marine biofouling Invertebrates. J. Proteome Res. 8, 2708–2719.
Timmins-Schiffman, E., O'Donnell, M.J., Friedman, C.S., Roberts, S.B., 2013. Elevated pCO_2 causes developmental delay in early larval Pacific oysters, *Crassostrea gigas*. Mar. Biol. 160, 1973–1982.
Tomanek, L., 2011. Environmental proteomics: changes in the proteome of marine organisms in response to environmental stress, pollutants, infection, symbiosis, and development. Ann. Rev. Mar. Sci. 3, 373–399.
Tomanek, L., Zuzow, M.J., Ivanina, A.V., Beniash, E., Sokolova, I.M., 2011. Proteomic response to elevated $_pCO_2$ level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. J. Exp. Biol. 214, 1836–1844.