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Effect of increased pCO_2 on early shell development in great scallop (*Pecten maximus* Lamarck) larvae

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Abstract

As a result of high anthropogenic emission of CO_2, partial pressure of carbon dioxide (pCO_2) in the oceans has increased causing a drop in pH, known as ocean acidification (OA). Numerous studies have shown negative effects on marine invertebrates, and that the early life stages are the most sensitive to OA. We studied the effects on embryo and larvae of great scallop (*Pecten maximus* L.), using mean pCO_2-levels of 477 (ambient), 821, 1184, and 1627 ppm. OA affected both survival and shell growth negatively after seven days. Growth was reduced with 5–10 % when pCO_2 increased from ambient 477 ppm to 1627 ppm, and survival based on egg number was reduced from 40.4 % in the ambient group to 10.7 % in the highest pCO_2-group. Larvae/embryos stained with calcein one day after fertilization, showed fluorescence in the newly formed shell area indicating calcification of the shell already at the trochophore stage. Shell hinge deformities were observed at elevated pCO_2-levels in trochophore larvae after two days. After seven days, deformities in both shell hinge and shell edge were observed in veliger larvae at elevated pCO_2-levels. Although the growth showed a moderate reduction, survival rate and increased amount of deformed larvae indicates that *P. Maximus* larvae are affected by elevated pCO_2 levels within the range of what is projected for the next century.

1 Introduction

The anthropogenic emission of CO_2 has lead to an increase in partial pressure of carbon dioxide (pCO_2) in the oceans and a decrease in pH (ocean acidification, OA). Caldeira and Wickett (2003) showed through modelling that there may be a rapid decline in pH in surface ocean waters over the next 100 yr of as much as 0.4 units (a doubling of H^+-ion concentration). Over the next 50–100 yr the pCO_2values are projected to reach 700 ppm, corresponding to a pH of about 7.85 (Zondervan et al., 2001; IPCC,

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2001). However, spatial and seasonal variations may cause values to extend beyond these average projections, to 1000 ppm and pH 7.4 (Caldeira and Wickett, 2003).

Depending on their distribution and habitats, marine organisms are exposed to various levels of pH fluctuations. In general deep sea species lives in a more stable environment compared to shallow living species that experiences both seasonal and daily fluctuations in physio-chemical water parameters. In areas with natural high CO_2-supply (up-welling of deep sea CO_2-rich water, volcanic carbon dioxide vents) marine organisms are exposed to seawater pCO_2 values as high as 2000 ppm, corresponding to pH 7.4–7.5 (Hall-Spencer et al., 2008; Thomsen et al., 2010). Some organisms can experience even lower pH-values in nature as shown for the oyster *Ostrea chilensis* (Chaparro et al. 2009). During valve closure and isolation of the brood chamber, the brooded veligers were exposed to pH-values as low as 7.0.

Although many marine organisms shows a high tolerance to variations in pH, several experimental studies conducted over the last decade have shown negative effects of OA, especially on calcifying organisms (Kroeker et al. 2010). Effects of elevated pH levels are shown on a range of marine invertebrates, (embryonic and larval development in marine molluscs – Byrne, 2012; Ericson et al., 2010; Gaylord et al., 2011; Gazeau et al., 2011; Kurihara, 2008; Kurihara et al., 2007, 2009; Waldbusser et al., 2010; and effect on other invertebrates – Bechmann et al., 2011; Dupont et al., 2010; Hoegh-Guldberg et al., 2007; Pane and Barry, 2007). Especially larval stages of bivalves seem to be extremely sensitive to enhanced levels of pCO_2 (Fabry et al., 2008; Talmage and Gobler, 2009, 2010, 2011), and most studies have shown decrease in survival, shell growth, and normal larval development. Some studies have also shown a negative effect on shell thickness and strength (Chaparro et al., 2009; Gaylord et al., 2011; Welladsen et al., 2010), calcification (Ries et al., 2009), lipid synthesis (Talmage and Gobler, 2011), and energy stores (Dickinson et al., 2012). The extreme sensitivity of bivalve larvae may be connected to the carbonate composition of the larval shell. Weiss et al. (2002) showed that larval shells (3–13 days old) of two bivalve larvae contained amorphous calcium carbonate in addition to aragonite. The dissolution of

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amorphous calcium carbonate is more rapid than aragonite, and even more so than calcite, when pCO_2 increases.

Acidification studies indicate both within-population (Chan et al. 2011) and species specific variation in sensitivity (Kurihara et al., 2009; Ries et al., 2009; Talmage and Gobler, 2009; Gazeau et al., 2011). Thomsen et al. (2010) showed in a survey study that calcifying keystone species may be able to handle OA in the magnitude projected for the end of this century when food supply is sufficient. However, when Ries et al. (2009) investigated the effects of OA on a broad range of 18 benthic marine organisms, they found that net calcification rate increased in seven species indicating a highly species specific response to OA.

Waldbusser et al. (2010) showed that the negative effect of OA on calcification in a hard clam, *Mercenaria* spp., depended on size (390 µm–2.9 mm), and also that there was a difference in calcification rate between two populations of the hard clam. Differences in populations were also shown by Walther et al. (2010) for larvae of the spider crab *Hyas araneus* from different latitudes (Svalbard and Helgoland). These studies indicate that genetics also may play a key role in the success of coping with OA.

The numerous reports on effects of OA are slowly building up a picture of how marine coastal ecosystems may be affected (biodiversity, functioning and service) in the future. It is crucial to gain more knowledge about the effects on marine organisms in order to get realistic projections of future changes of the marine food web. Together with mussels and oysters, several scallop species are also economically important in global aquaculture production (Bourne, 2000; FAO, 2010), in addition to be important calcifyers in the marine ecosystem. A negative effect on early life stages may not only be detrimental to recruitment and endanger the species survival, but also result in economic loss due to a collapse in global shellfish aquaculture production (Gazeau et al., 2010).

The great scallop (*Pecten maximus* Lamarck) is distributed south from the Iberian Peninsula up to Lofoten in Norway in the north and found in highest abundances at 20–40 m depth in shell sand areas. The larvae are pelagic for about 15–32 days at

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temperatures of 15–18 °C (Comely, 1972; Le Pennec et al., 2003). The great scallop is a high-valued sea food product and has been cultured for more than 25 yr in Norway. Extended research effort during the same period makes it an excellent candidate for OA studies (Andersen et al., 2011).

Most studies on mollusk larvae focus on effects on shell formation (Kurihara et al., 2007, 2009; Kurihara, 2008; Ericson et al., 2010; Waldbusser et al., 2010; Byrne, 2012; Gaylord et al., 2011; Gazeau et al., 2011). The shell that starts to evolve at the end of the trochophore stage consists of proteins (Bellioli et al., 1993; Casse et al., 1998). In pectinids it is calcified within less than a day, at the the veliger stage (Cragg, 2006). If calcification is the actual process being affected, effects on the shell formation should be detected only after the stage when the calcification starts. The onset of calcification can be determined by the use of an epifluorescent dye, calcein that bonds to calcium in metabolic active individuals (Körbl and Vydra, 1958; Day et al., 1995; Lucas et al., 2008; Chaparro et al., 2009). Calcein has little or no effect on mollusc larvae survival or growth (Moran, 2000; Moran and Marko, 2005; Chick, 2010; Fitzpatric et al., 2010).

The aim of the present study was to report the effect of elevated pH levels on scallop *Pecten maximus* Lamarck larvae survival, growth and shell development from fertilized eggs to seven days old veligers. Three acidification levels were used based on the predicted drop of 0.5 units from today to year 2250 (IPCC, 2001; Caldeira and Wickett, 2003). The exact initiation of calcification in *P. maximus* larvae is still unknown, thus calcein staining of larvae was tested as a method to elucidate this question.

2 Material and methods

Local broodstock were collected in January 2012 from the outer Hardangerfjorden, located on the south-western coast of Norway. The scallops were conditioned for eight weeks at 12.0–12.5 °C and fed 13–15 cells μL^{-1} of a standard diet containing *Isochrysis galbana* (Tahitian), *Pavlova lutheri* and *Chaetoceros mulleri* (Andersen et al., 2011). Spawning was induced on 21 March by increasing the temperature to 16–17 °C, and

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eggs were cross fertilized with a mixture of sperm from 2–3 individuals. Egg number was determined by counts in 10 subsamples of 50 µL, and 13 fertilized eggs mL^{-1} were incubated in 38 L exposure tanks at ambient pH 7.98 (control) and mean pH-levels of 7.71, 7.64, and 7.51 (table 1). Mean temperature (± sd) was 15.6±0.3 °C (n = 495). The tanks were circular with slightly conical bottom, resulting in a depth difference of 22 mm between tank center and edge. The inner tank diameter was 500 mm, and water depth was 226–257 mm at the tank edge. Four replicates were used per pH-level, and the flow rate was set to 10 $L\,h^{-1}$ per exposure tank. To prevent escape of embryos, water flow in the experimental tanks was turned off over night at incubation of the fertilized eggs. A slow flow (< 10 $L\,h^{-1}$) was re-established after 10.5 h, and then increased to 15 $L\,h^{-1}$ (flow meter readings) after another 3.3 h. Slow bubbling was introduced at the tank bottom through a perforated plastic tube. Larvae were not fed during the experimental period.

Seawater at different pH levels was produced by mixing seawater with an acid stock solution of pH 5.80, made from mixing CO_2-gas and seawater with an ambient pH of 7.95. The pH in each mixing tank was continuously adjusted to pre-set pH levels by addition of stock solution with dosage pumps (IWAKI) controlled by feedback from pH electrodes to pH transmitters (Endress and Hauser). The different water qualities were distributed by gravity to the exposure tanks via four high mounted header tanks. The water level in header and mixing tanks was kept constant by flotation valves, and water was pumped in a loop between the mixing tanks and header tanks. Water flow in the loop was much higher than the flow from header tanks to the exposure tanks, in order to keep the pH-level constant in the header tanks.

Exposure tanks were illuminated continuously by fluorescent tubes (Philips TL-D 18w/830) 110 cm above the tanks. Light was dispersed through an opaque sheet of polycarbonate 70 cm above the exposure tanks.

The pH-level in each exposure tank was measured daily in a 100 ml sample using a Mettler Toledo, and total alkalinity (TA) was analyzed in four replicate samples on 9, 15 and 26 March 2012 by a Titralab, Radiometer. Salinity was measured by

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a WTW LF330 Conductivity meter, and temperature recorded every hour using a four detector (one in air and three in exposure tanks) EBI – 1 Ebro 4 temperature logger. The pCO_2 (ppm) corresponding to the pH-values were calculated from the daily average temperature (°C) based on hourly recordings, salinity, TA and pH using the macros in Ernie Lewis' "CO2SYS.BAS" Basic Program (Pierrot et al., 2006).

Larvae samples were taken after 1, 2, and 6 days after carefully stirring from bottom to top using a plunger. A 50 mm diameter pipe was lowered to the bottom and both ends were plugged before the pipe was pulled out of the water, collecting a volume of 350 mL. To determine timing of shell calcification, larvae from the control tanks were stained overnight (18–20 h) from day 1 to day 2. At day 2 a total volume of 700 mL were withdrawn to classify larval deformities. Sampling was carried out so that the total volume withdrawn from each replicate was identical. When terminating the experiment after 7 days, tanks were drained and a total sample from each replicate were collected and preserved to determine final shell size, survival, and classify deformities.

When collected for preservation, embryos and larvae were retained on 35 µm mesh screen and preserved in 4 % formalin (diluted from 36.5 % formaldehyde solution, Sigma-Aldrich, cat. no. 33220) in PSB for two hours, rinsed twice in 70 % ethanol and then kept in 70 % ethanol until the ratio of developmental stages were investigated. The formalin was made from mixing 8.14 g di-sodium hydrogen phosphate dehydrate ($Na_2HPO_4 * 2H_2O$, Merck 1.06580.1000), 4.00 g sodium di-hydrogen phosphate monohydrate ($NaH_2PO_4 * H_2O$, Merck 1.06346.1000) with 100 mL of 37 % formalin in 900 mL distilled water.

For staining of calcium, 20 ml calcein stock solution was added to the 350 mL of seawater with larvae, giving a final calcein concentration of 25 mg L^{-1}. A calcein stock solution was made from mixing 1 g calcein (Sigma C0875-5G) in 1 L distilled water adjusted to pH 7.0 using a Sodium bicarbonate ($NaHCO_3$, Merck 1.06329.1000) solution of 8.4 g in 500 ml distilled water (Moran, 2000; Moran and Marko, 2005; Fitzpatrick et al., 2010).

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Preserved and live (calcein stained) larvae were photographed using a Canon EOS 5D Mark II camera attached to an Olympus BX60 microscope. Final shell length (parallel to the hinge) and height (perpendicular to the hinge) were measured in 21–60 individuals to the nearest 0.1 µm from printed photographs. Survival was estimated by concentrating all day 7 larvae in 25–110 ml and count the number in 10 subsamples of 50 µL. Preserved larvae photographed at 100 magnifications using bright field, were classified according to shell shape and counted. Number of individuals classified on day 2 (trochophores) was 76–150, and 46–178 individuals were examined on day 7.

Live, calcein stained larvae were photographed at 10 × 40 magnification using an U-MSWB2 cube filter (excitation 420–440, emission 475) and UV light to show yellow-green fluorescence when calcein bonded with calcium (Moran, 2000; Moran and Marko, 2005; Fitzpatrick et al., 2010).

Statistica version 11 (Statsoft Inc.) were used to run statistical tests. Shapiro-Wilk's W test of normality was used, and normality was also checked by inspection of box-plots. All percentage data were arc-sin transformed to meet the assumption of normality. Effects of pCO_2 on all parameters were tested using a simple linear regression. In addition, one-way ANOVA was used to test differences in survival between groups, to find groups different from ambient.

3 Results

3.1 Variation in pCO_2-values

The mean pH-values and the corresponding mean calculated pCO_2 are given in Table 1. The pCO_2-values for the elevated groups showed a sharp decrease during the first night due to a discontinuation in seawater flow in the exposure tanks (Fig. 1). This drop in pCO_2 (delta, Δ, pCO_2) increased with increasing pCO_2-levels, and fell from a mean of 2534 to 1225 ppm (ΔpCO_2 of 1309 ppm) in the highest pCO_2-group. The ambient group showed only an insignificant ΔpCO_2 of 20 ppm. Naturally, the variation

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in pCO_2-values given as the coefficient of variation (CoV), increased with increasing pCO_2-levels, and was higher between days for any given replicate than between replicates on a given day (Table 1).

3.2 Survival and shell size

The survival ratio of day 7 veligers based on the initial number of eggs, reared at ambient pCO_2 was 40.4 % and decreased at higher pCO_2-levels (Fig. 2). At the highest pCO_2-level the survival was 10.7 %. The relationship between survival and pCO_2 was linear ($p < 0.001$), and one-way ANOVA showed that survival at ambient pCO_2 was higher than at 821 and 1627 ppm ($p < 0.003$), but not at 1184 ppm ($p = 0.055$). Shell length and height seven days after incubation (day 7) were both negatively affected by an increase in pCO_2-level (Fig. 3a) as they decreased by 10 and 5 %, respectively, when pCO_2 increased from 477 ppm (ambient) to the highest $pCO2$ of 1627 ppm. The shell ratio length : height was not affected, and varied between 1.28 and 1.32 and (Fig. 3b).

3.3 Shell deformities

On day 2 an average of 85 % (82–88 %) of examined individuals had developed a shell, and only 1 % had not developed further from egg stage. The observed shell deformities were seen on the hinge and edge. The hinge deformity was shown as an indentation of the hinge centre, making the hinge convex rather than straight (Fig. 4a, b). The edge deformity was either an indentation of the shell margin centre (Fig. 4c) or on the side (Fig. 4d). Some veliger larvae were observed to have both types of deformities. Preserved larvae were then classified in four groups according to shell shape, and counted: (1) normal, (2) edge deformities, (3) hinge deformities, and (4) both edge and hinge deformities. Trochophore larvae were only classified in group 1 and 3, since shell edge was not visible until the shell valves covered the whole larva (Fig. 4a). In day 7 veligers both hinge and edge deformity was observed.

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Out of the shelled individuals, an average of 28–68 % in the different exposure groups on day 2 had developed a normal-looking straight hinge. On day 7 the ratio of normal hinges was higher, 68–95 % for the different $pCO2$-groups. The ratio of larvae showing a normal hinge was negatively affected by pCO_2 on both days ($p < 0.001$ for both), and the lowest ratio was found at the highest pCO_2-level (Fig. 5). Ratios of larvae classified with different shell deformities or none on day 7 are shown in Fig. 6. On day 7 shell edge deformities were 30.5–57.4 % for the different pCO_2-groups, but there were no linear relationship between edge deformities and pCO_2 ($p = 0.941$). The mean ratio of normally shaped larval shells was negatively affected by increasing pCO_2, and decreased from 63.5 to 27.8 % with an increase in pCO_2 from 477 to 1627 ppm. The ratio of larvae with hinge deformities, and both hinge and edge deformities, increased significantly when pCO_2 increased. Variation within replicates was high in all deformity classes as the coefficient of variation (CoV) ranged within 5–70 % for all pCO_2-levels.

3.4 Calcification

Trochophore larvae (Fig. 7a, b, i) stained with calcein overnight (day 1–2) showed green fluorescence when exposed to UV-light (Fig. 7a, b, ii, iii). The fluorescence was highly concentrated in a small area on each valve, closer to the hinge than the shell margin centre. Also, there was a weak fluorescence from the total shell area, indicating that calcification of the protein shell had already started between day 1 and day 2.

4 Discussion

4.1 Variation in pCO_2-level

The rapid decrease in pCO_2-values during the first 10 h at the three exposure levels were more pronounced at the highest pCO_2-level. The increase was due to both the discontinuation in seawater flow during the first night, and gas exchange at the water surface. The drop in pCO_2-values (ΔpCO_2) at the beginning of the experiment

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increased with increasing pCO_2-level. The negative effect of higher pCO_2-levels seen in this study may therefore include an effect of higher ΔpCO_2.

4.2 Survival and shell size

The survival ratio of day 7 veligers for the control group reared at ambient pCO_2 in this study was 40.4 %. In comparison, the survival of day 3 veliger in a Norwegian hatchery was 25–46 % with an average of 36.7 % of 12 broodstock groups (Magnesen et al. 2006). This indicates that the larval group used in the present study was viable and healthy. When the pCO_2-level increased from ambient 477 to 1627 ppm, survival of the day 7 larvae was reduced from 40 % to 10 %. During the same period larvae shell height and length decreased by about 10 % when pCO_2 increased from ambient to 1627 ppm.

The reduction in survivorship was much higher compared to larvae of hard clam (*Mercenaria mercenaria*), bay scallop (*Argopecten irradians*) and Eastern oyster (*Crassostrea virginica*) (Talmage and Gobler, 2009). The highest reduction in survival on day 6–8 for these larvae was 20 % for the oyster larvae at 64 and 150 Pa CO_2 (pH 7.83–7.85 and pH 7.48–7.50, respectively) when ambient conditions was 36 Pa (pH 8.02–8.08). The reduction in shell growth found in the present study was in accordance with previous studies (Gaylord et al., 2011; Kurihara et al., 2007, 2009). Gaylord et al. (2011) found that when pCO_2 increased from ambient 380 to 970 ppm the shell area of mussel larvae (*Mytilus californianus*) at day 5 and day 7 was reduced by 7 % and 5 %, respectively. Kurihara et al. (2007) showed the shell length of oyster (*C. gigas*) larvae after 48 h to be ca. 20 % smaller at a pCO_2 of 2268 µatm (pH 7.42) than at the ambient 348 µ atm (pH 8.21). Kurihara et al. (2009) also found that shell length of *Mytilus galloprovincialis* larvae was reduced by ca. 20 % after six days when pH was reduced from the ambient 8.05 (14.3 µmol CO_2 kg^{-1}) to 7.43 (83.3 µmol CO_2 kg^{-1}). A study on abalone larvae (*Haliotis kamtschatkana*) showed that both survival and growth were reduced with 40 % at day 8 when pCO_2 was elevated from ambient 400 ppm to 800 ppm (Crim et al., 2011).

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On the contrary, Pacific oyster (*Crassostrea gigas*) embryos exposed to 1020 and 2171 ppm pCO_2 for 72 h until D-veliger stage did not show significant effects on % hatching (development to D-veliger stage), although values were lowest for larvae exposed to the highest pCO_2 level (Gazeau et al., 2011). Neither did (Gazeau et al. 2011) find significant effects on shell length or shell area for the oyster embryos, although values for three replicates were lowest for larvae exposed to the highest pCO_2.

Negative environmental stress may also be a result of unfavourable rearing environment, such as small culture volumes, a stagnant rearing system, and discontinuous feeding (Andersen et al., 2011). A large part of the work undertaken to investigate effects of OA on marine mollusc larvae have been carried out in volumes of 50 mL–10 L (Kurihara et al., 2007, 2009; Talmage and Gobler, 2009, 2010, 2011; Gaylord et al., 2011; Gazeau et al., 2011). As mentioned by Wernberg et al. (2012) it may be problematic that so many experimental studies of ocean acidification effects are undertaken in relatively small volumes. Although the larvae are small in size, small rearing volumes may not give sufficiently stable rearing environment, and result in negative environmental stress. Exposure tanks in the present study contained 38 L and this may be too small to keep variations in the rearing environment at a low level. The results for the control group, however, indicate that the variations were within an acceptable limit.

Thomsen et al. (2010) showed that calcifying keystone species may be able to handle the change in seawater chemistry projected for the end of the century as long as food supply is sufficient. Even if our experiment lasted only for seven days after fertilization, lack of food is known to affect the energy status of scallop larvae after only three days (Andersen et al., 2012). The control group at ambient pH seemed to develop normally, however, the lack of food can be regarded as a stress factor that may push the larval energy status towards a point where the effects will be visible as slower growth, increased mortality and increased shell deformity. The change in water chemistry in the lower pH-groups to a less favourable level may be the extra stress that pushes the larvae past this point of visible damage.

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The reduction in larvae shell growth found in the present study is at the lower end of the scale compared to the referred studies. This may indicate that *P. maximus* shell development is not the most sensitive to OA compared to other bivalves. However, the high reduction in survival may indicate that other physiological processes are highly affected by OA. The duration of the study was only seven days, thus keeping the scallop larvae in the same conditions throughout the larval cycle will elucidate how OA over a longer term will affect growth and survival and also the ability of going through the critical metamorphosis stage.

4.3 Shell deformities

The present study observed negative effects of OA on normal larval shell development. Watson et al. (2009) found a similar trend in 8 day old Sydney rock oyster *Saccostrea glomerata* larvae. Using scanning electron microscopy they found abnormalities on the shell surface at low pH (7.8 and 7.6). On the contrary, Bechmann et al. (2011) found no effect of pH 7.6 at 10 °C on abnormality to the D-shell stage of mussel larvae compared to ambient pH 8.1.

The two shell deformity classes observed in larvae in the present study have previously been described by His et al. (1997) as "convex hinge" and "indented shell margins". Convex hinges were also shown by Kurihara et al. (2007) both in *C. gigas* larvae reared under control conditions of pH 8.21 (348 µ atm pCO_2) and under exposed conditions of pH 7.42 (2268 µ atm pCO_2), although it was not given any attention in the study to deformity in the control group. All larvae treated with the high pCO_2-level had an irregular-shaped shell and were considerably smaller than the untreated larvae. Kurihara (2008) show three types of deformities in day 6 *C. gigas* veligers: indented shell margin, protruding mantle and convex hinge, as described in His et al. (1997). Protruded velum was also described as a deformity or abnormality by His et al. (1997) and Spangenberg and Cherr (1996). This classification was not included in the present study as it may have been caused by the preservation method.

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The higher ratio of normally developed hinges on day 7 compared to day 2 may be a result of increased mortality of abnormal larvae from day 2 to 7. The variation between replicates in shell deformities on day 7 independent of pCO_2 indicates that factors in the rearing environment others than pCO_2 may have affected shell development. Also, larvae in the control group had edge deformities, unrelated to OA. In the present study the ratio of normally developed larvae on day 7 was 64 % in the control group. The common ratio of normal shell development in *P. maximus* larvae has been poorly described and is not known. In a control group of oyster (*C. gigas*) larvae Kurihara et al. (2007) described that 68 % developed into normal D-shaped veligers, and 72 % of these larvae had fully mineralized shells after 48 h. This was explained as rather low values for the control group, and was explained as a result of using gonadal stripping to produce the embryos. In a study on *M. galloprovincialis* Kurihara et al. (2009) reported abnormal morphology in < 1 % of control larvae.

Increased deformity in mollusc larvae seems to be a general expression of environmental and genetic impacts, and deformities are seen in untreated groups in OA studies. This may relate to factors in the rearing environment, caused by small rearing volumes, stagnant systems, addition of antibiotics and pulse feeding. These characteristics are common for most larval experiments reported (Kurihara et al., 2007, 2009; Talmage and Gobler, 2009, 2010, 2011; Waldbusser et al., 2010; Gaylord et al., 2011; Gazeau et al., 2011,).

Assessing larval deformity has also been used to describe the effects of pollutants (trace metals, bleached-kraft mill effluent) quantitatively (Conroy et al., 1996; Spangenberg and Cherr, 1996; His et al., 1997). Spangenberg and Cherr (1996) reported that approximately 10 % of the larvae had abnormal larval shell calcification and embryo morphology in the mussel *M. californianus* as a response to Ba exposure.

Self fertilization may also cause an increase in shell deformities. Concha et al. (2011) describe that malformed larvae of the northern scallop *A. purpuratus* were negatively correlated with the selfing rate and the mean temperature of the culture. Shell deformity has also been shown to be higher (14.1 %) in veliger larvae from inbreeding of the

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mollusk Pacific abalone (*Haliotis discus hannai*), compared to outbreed groups (5.2 %) (Kobayashi and Kijima, 2010). The deformities shown in Kobayashi and Kijima (2010) included both abnormally small shells and an inability to retract the larval body into the shell, similar to deformities shown in Crim et al. (2011) when larvae of the northern abalone *H. kamtschatkana* were exposed to increasing OA.

In the present study, shell deformities cannot be explained by self fertilization as we cross fertilized the eggs. Also, the rearing environment was the same in all replicates except for the difference in pH. No feed or antibiotics were added and the water was not polluted. Although the ratio of deformed larvae varied highly between the treatments, the results show a tendency of more shell deformities with increasing pCO_2-level.

4.4 Calcification

The negative effect of OA on larval shell formation may be caused by dissolution of the calcified shell, a decrease in calcification rate or the organism's ability to calcify or a combination of all. Kurihara et al. (2007) reported that some *C. gigas* larvae reared under exposed conditions of pH 7.42 showed limited or no mineralization of the shell when observed under polarized light in a microscope. Orr et al. (2005) showed shell dissolution in a live pteropod from the subarctic Pacific that was kept in water undersaturated with respect to aragonite for 48 h. Nienhuis et al. (2010) suggested that declines in shell weight gain observed in live snails exposed to increasing pCO_2 were due to increased dissolution of existing shell material, rather than a decrease in shell production rate. This was based on a shell weight loss per day in empty shells that was parallel to the decrease in shell weight gain per day in live snails with increasing pCO_2 levels.

Range et al. (2011) found no negative effects of increased pCO_2 and reduced pH on net calcification, size or weight of the clam *Ruditapes decussatus* juveniles in a 75 days long experimental study. However, the lack of OA effects was explained by the buffering effect of naturally elevated total alkalinity of local seawater.

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In the present study we confirmed that calcein bonded to the calcium in 1–2 day old trochophore larvae. However, we were unable to determine the exact time of initiation of calcification in the larvae. Either the calcification process had started before larvae were collected or it was initiated during the 18–20 h staining period. Staining embryos and larvae frequently (e.g. every 5 h) for a shorter period (e.g 3 h) from day 0 to day 2 may reveal more details on the onset of the calcification process.

4.5 Bivalves in a high CO_2 world

The rather severe effects of elevated future pCO_2 levels on a number of mollusc larvae seem undisputable. Talmage and Gobler (2009) showed that preindustrial levels of pCO_2, 250 ppm, improved the survival for larval *M. mercenaria* and *A. irradians* from day 7 to 36 compared to the results for the control group at ambient levels (390 ppm). This indicate that the pCO_2 levels of today may already affect bivalve larvae negatively compared to preindustrial levels, and that negative effects of OA is happening today rather than in the future. Also, OA should be seen in combination with the increase in global ocean temperature (NOAA 2010). Effects of a temperature increase may amplify the OA effects or reduce them. Talmage and Gobler (2011) showed that the negative effects of elevated pCO_2 and temperature on survival, growth and lipid synthesis in larval *M. mercenaria* and *A. irradians* were additive. Similarly, elevated CO_2 levels caused a narrowing of the temperature tolerance window in adult spider crab, *H. araneus* (Walther et al., 2010).

Long term studies on larvae of the great scallop *P. maximus* are needed to elucidate the effect of OA throughout the larvae stages, metamorphosis and settlement. Such studies should involve assessment of energy budget, take place in facilities that provides the animals with sufficient space and nutrition for a normal development, and include studies of temperature effects. Effects on important processes for survival, such as immune defence functions, feeding rate, and shell strength should also be included. More studies using calcein at the earliest larvae stages (day 0–2) are needed

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to increase the knowledge about the onset of larval shell calcification process that is crucial to the existence of calcifying organisms.

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Table 1. The pH-values (mean ± sd) from daily measurements for seven days in four (*three) tanks per group, and the corresponding calculated pCO_2. Ambient pH was 7.98. CoV (sd as % of mean) is shown for variation between days (d) for given replicates, and between replicates (r) for given days.

pH	pCO_2 (ppm)	CoV (d)	CoV (r)
7.98 ± 0.01	477 ± 9	1.1–2.2	0.0–2.2
7.78 ± 0.05	821 ± 108	12.5–15-9	1.3–11.4
*7.64 ± 0.07	1184 ± 222	18.9–20.6	1.4–11.4
7.51 ± 0.10	1627 ± 429	23.3–33.7	1.2–13.8

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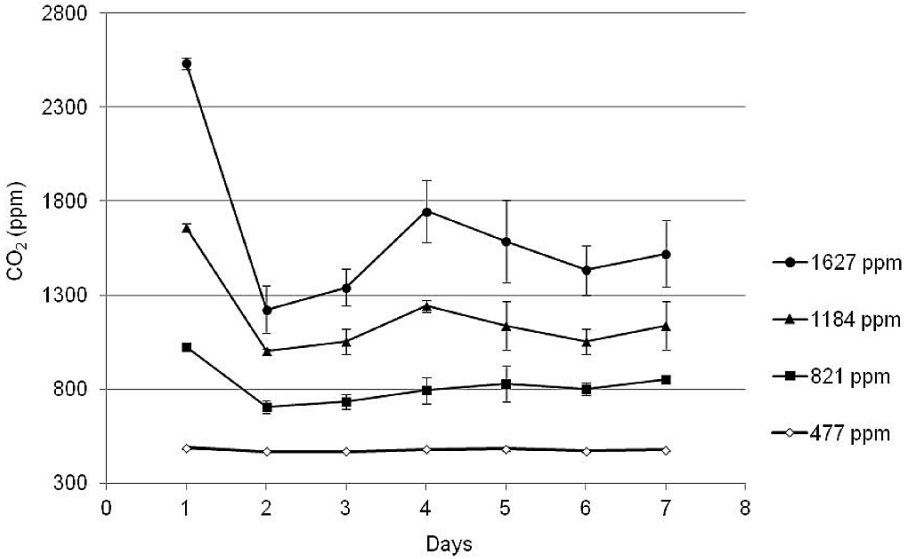

Fig. 1. Mean daily pCO_2 in the control (ambient, 477 ppm) and three elevated pCO_2-groups, 821, 1184 and 1627 ppm. Error bars are sd, n = 4 except for the 1184 ppm-group (n = 3).

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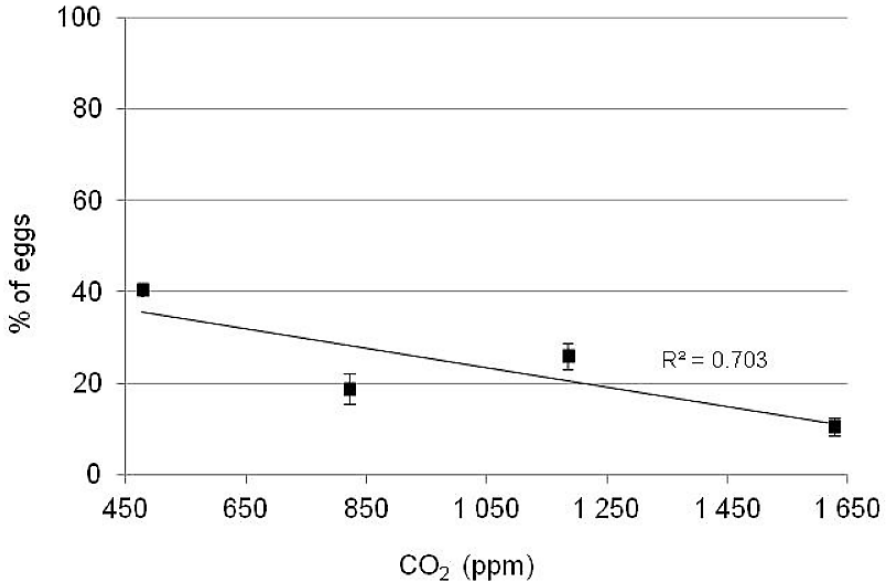

Fig. 2. Survival of day 7 larvae based on the number of eggs incubated. Values are mean (n = 4, $^{*}n$ = 3), error bars are sd.

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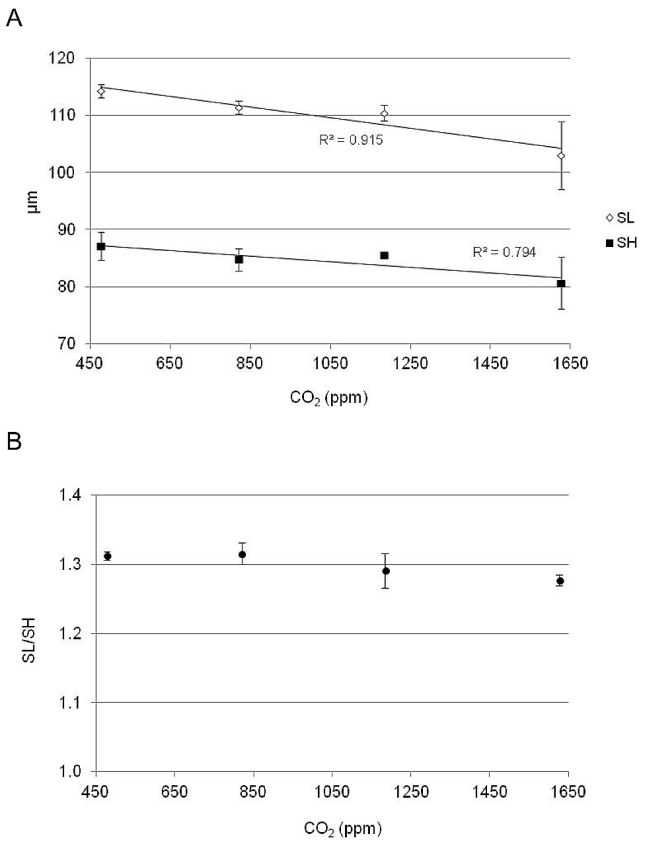

Fig. 3. Shell size **(A)** given as shell length (SL) and shell height (SH), and the ratio **(B)** shell length : shell height (SL/SH) for day 7 veligers at different pCO_2 levels. Values are mean, $n = 4$ except for the 1184 ppm-group ($n = 3$), and error bars are sd. Linear regressions are only drawn for significant slopes.

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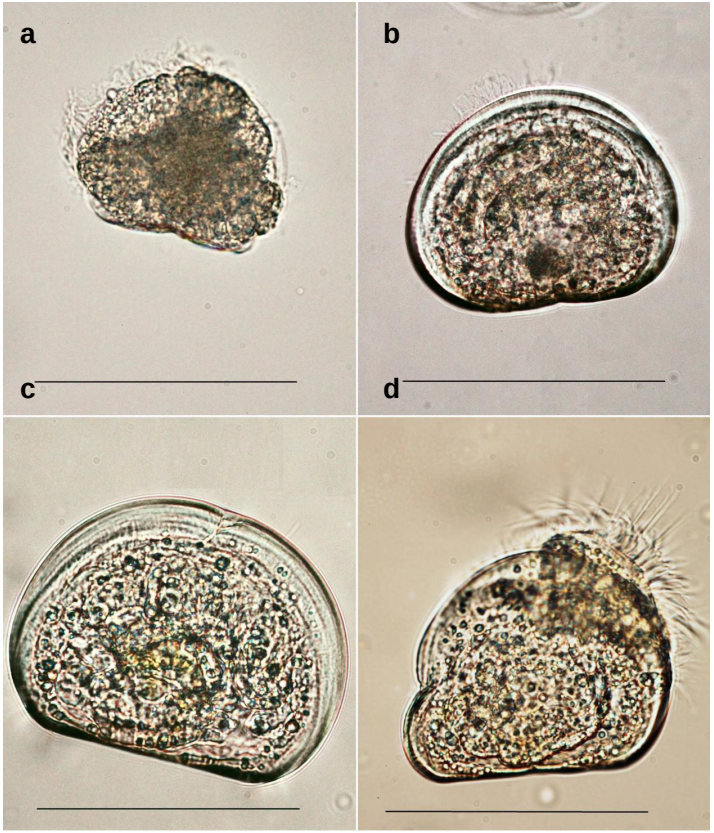

Fig. 4. Shell deformity classes observed in *Pecten maximus* larvae. Hinge deformity in formalin preserved larvae: **(a)** day 2 trochophore larva; **(b)** day 3 veliger larva. Edge deformity in live larvae: **(c)** shell edge centre of a day 7 veliger larva; and **(d)** shell edge side of a day 6 veliger larva. The scale bar is 50 µm in **(a)** and **(b)**, and 100 µm in **(c)** and **(d)**.

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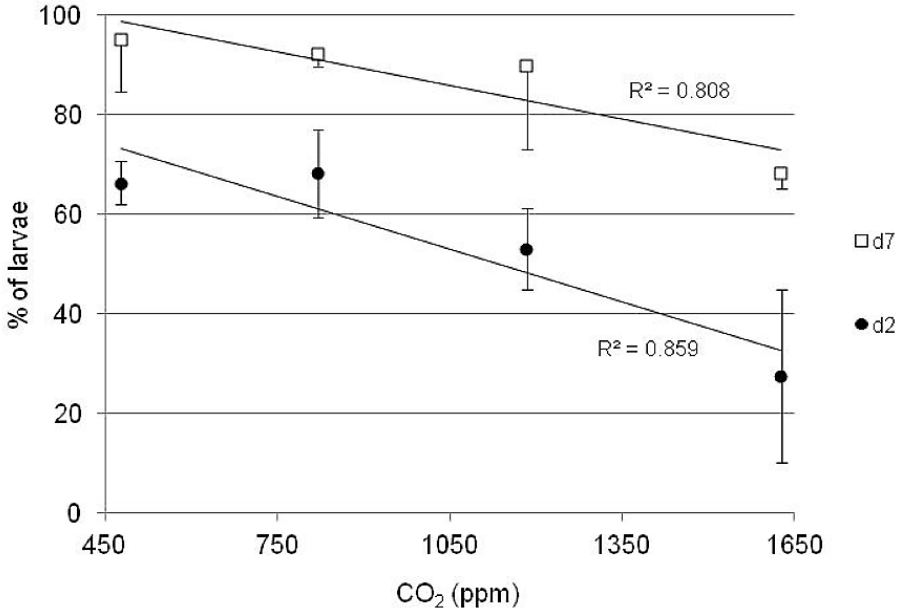

Fig. 5. Ratio of larvae (%) with a normal hinge on day 2 (d2) and day 7 (d7) based on the total number, at four different levels of pCO_2 (ppm). Values are mean, $n = 4$ except for the 1184 ppm-group on day 7 ($n = 3$), and error bars are sd.

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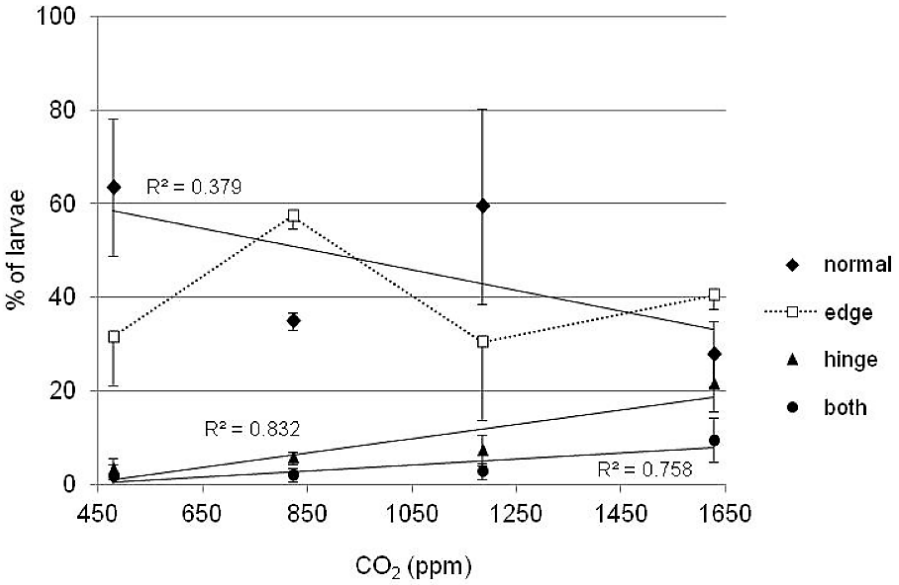

Fig. 6. Ratio (%) of normal shells and different shell deformities on day 7. The group "both" includes both edge and hinge deformities that are not included in either "edge" nor "hinge". Values are mean, $n = 4$ except for the 1184 ppm-group ($n = 3$), and error bars are sd. Linear regressions are only drawn for significant slopes.

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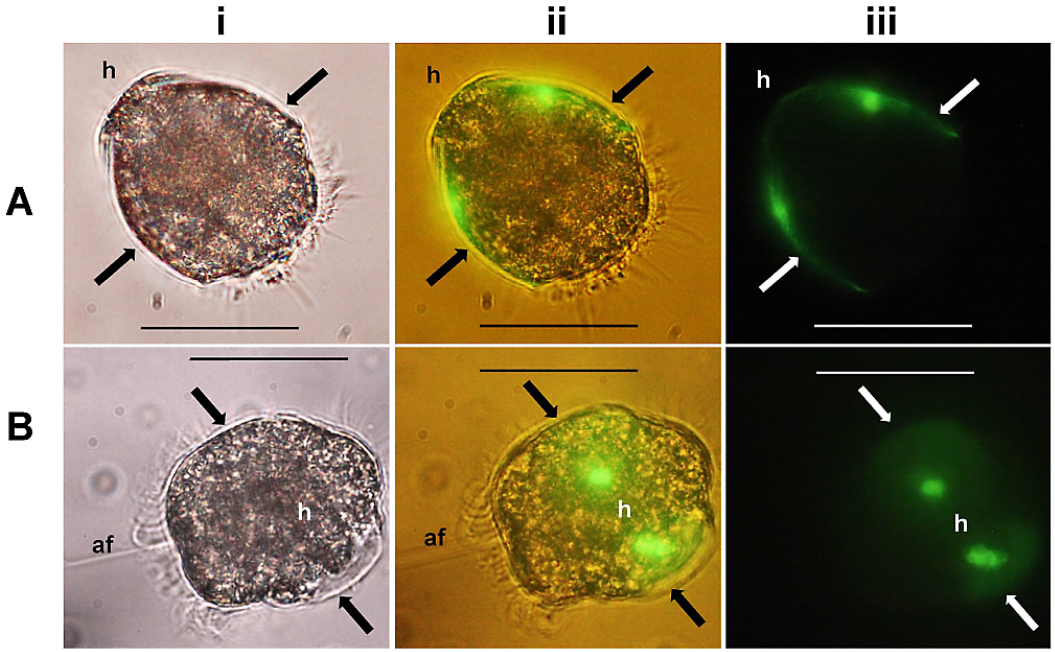

Fig. 7. Stereomicroscopic images of live trochophore larvae stained with calcein from day 1 and overnight (18–20 h). **(A)** a side view with the hinge area (h) oriented towards the upper left corner and a newly formed shell valve on each side (arrows); **(B)** shows the hinge area (h) and early shell formation (arrow); using (i) bright field; (ii) bright field and UV-light; (iii) UV-light. Green colour in UV-light (fluorescence) shows areas containing calcium. The scale bar is 50 µm, and magnification is the same for all photographs.

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