Biogeosciences, 10, 6161–6184, 2013
www.biogeosciences.net/10/6161/2013/
doi:10.5194/bg-10-6161-2013

Biogeosciences Open Access

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

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Received: 25 January 2013 – Published in Biogeosciences Discuss.: 22 February 2013
Revised: 16 August 2013 – Accepted: 22 August 2013 – Published: 1 October 2013

Abstract. As a result of high anthropogenic CO_2 emissions, the concentration of CO_2 in the oceans has increased, causing a decrease in pH, known as ocean acidification (OA). Numerous studies have shown negative effects on marine invertebrates, and also that the early life stages are the most sensitive to OA. We studied the effects of OA on embryos and unfed larvae of the great scallop (*Pecten maximus* Lamarck), at pCO_2 levels of 469 (ambient), 807, 1164, and 1599 µatm until seven days after fertilization. To our knowledge, this is the first study on OA effects on larvae of this species. A drop in pCO_2 level the first 12 h was observed in the elevated pCO_2 groups due to a discontinuation in water flow to avoid escape of embryos. When the flow was restarted, pCO_2 level stabilized and was significantly different between all groups. OA affected both survival and shell growth negatively after seven days. Survival was reduced from 45 % in the ambient group to 12 % in the highest pCO_2 group. Shell length and height were reduced by 8 and 15 %, respectively, when pCO_2 increased from ambient to 1599 µatm. Development of normal hinges was negatively affected by elevated pCO_2 levels in both trochophore larvae after two days and veliger larvae after seven days. After seven days, deformities in the shell hinge were more connected to elevated pCO_2 levels than deformities in the shell edge. Embryos stained with calcein showed fluorescence in the newly formed shell area, indicating calcification of the shell at the early trochophore stage between one and two days after fertilization. Our results show that *P. maximus* embryos and early larvae may be negatively affected by elevated pCO_2 levels within the range of what is projected towards year 2250, although the initial drop in pCO_2 level may have overestimated the effect of the highest pCO_2 levels. Future work should focus on long-term effects on this species from hatching, throughout the larval stages, and further into the juvenile and adult stages.

1 Introduction

The increase of anthropogenic CO_2 emissions since the industrial revolution has lead to an increase of carbon dioxide (CO_2) concentration and a decrease in pH in the oceans termed as ocean acidification (OA). Over the next 50–100 yr the CO_2 values are projected to reach 700 ppm (Zondervan et al., 2001; IPCC, 2001). Caldeira and Wickett (2003) showed through their geochemical model that with no reduction in fossil fuel emission of CO_2 into the atmosphere, 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). And within less than 250 yr the ocean surface pH may drop as much as 0.7 units, which may be the lowest pH value experienced during the last 300 million years (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 live in a more stable environment compared to shallow-living species that experience both seasonal and daily fluctuations in physio-chemical water parameters. In areas with natural high CO_2 supply (upwelling of deep-sea CO_2-rich water and volcanic CO_2 vents) marine organisms are exposed to seawater CO_2 values as high as 2000 ppm, corresponding to a pH of 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 show 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). The effects of elevated CO_2 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, 2008; Waldbusser et al., 2010; and effect on other invertebrates – Bechmann et al., 2011; Dupont et al., 2010; Jury et al., 2010; Comeau et al., 2009, 2010; Ries et al., 2009; Hoegh-Guldberg et al., 2007; Pane and Barry, 2007). Especially larval stages of bivalves seem to be extremely sensitive to enhanced levels of CO_2 (Fabry et al., 2008; Talmage and Gobler, 2009, 2010, 2011), and most studies have shown a 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; Waldbusser et al., 2010; Range et al., 2011), lipid synthesis (Talmage and Gobler, 2011), and energy stores (Dickinson et al., 2012). The high sensitivity of bivalve larvae may be connected to the carbonate composition of the larval shell. A mollusc's larval shell contains mainly aragonite, the most soluble form of crystalline calcium carbonate (Weiss et al., 2002; Kudo et al., 2010; Barros et al., 2013). The predicted undersaturation of aragonite in the world's oceans may have a great negative impact on these calcifying organisms (Orr et al., 2005; Raven et al., 2005).

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 (Raven et al., 2005). It is crucial to gain more knowledge about the effects on different marine organisms in order to get realistic projections of future changes of the marine food web. 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). Together with mussels and oysters, several scallop species are economically important in global aquaculture production (Bourne, 2000; FAO, 2010) in addition to being important calcifiers in the marine ecosystem.

The great scallop *Pecten maximus* Lamarck is distributed from the Iberian Peninsula in the south up to Lofoten in Norway in the north, and is found in highest abundances at 5–50 m depth in shell sand areas (Strand and Parsons, 2006). The larvae are pelagic for about 15–32 days at temperatures of 15–18 °C (Comely, 1972; Le Pennec et al., 2003). The great scallop is a highly valued sea food product, and has been cultured for more than 25 yr in Norway. The extended research efforts during the same period make it an excellent candidate for OA studies (Andersen et al., 2011). To our knowledge, only the study of Schalkhausser et al. (2012) has shown the effect of OA on *P. maximus*. They found a negative effect of OA on adult *P. maximus* shell-clapping performance and aerobic scope. However, no studies have focused on the effect of OA on *P. maximus* larvae. Studies on other scallop species as *Argopecten irradians*, *Placopecten magellanicus* and *Chlamys nobilis* show a negative effect of increased pH in most life stages, including fertilization, embryos, larvae, and juveniles (Desrosiers et al., 1996; Talmage and Gobler, 2009, 2010, 2011; Liu and He, 2012).

Most OA studies on mollusc larvae focus on effects on shell formation (Kurihara et al., 2007, 2008; 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 trochophore stage consists of proteins (Bellioli et al., 1993; Casse et al., 1998). Onset of calcification in the initial organic larval shell was detected during the trochophore larval stage in *Mercenaria mercenaria* and *Crassostrea gigas* larvae (Weiss et al., 2002). Casse et al. (1998) did not detect calcification in *P. maximus* trochophores, and according to Cragg (2006) it is likely that there is initially little or no calcification in pectinid larvae, but that the calcification starts within less than a day after the initial shell formation. 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, which binds to calcium in metabolically active individuals (Körbl and Vydra, 1958; Day et al., 1995; Lucas et al., 2008; Chaparro et al., 2009).

The aim of the present study was to report the effect of elevated CO_2 levels on scallop *P. maximus* Lamarck larvae survival, shell size, and shell development from fertilized eggs to 7-day-old veligers. The exact initiation of calcification in *P. maximus* larvae is still unknown, and thus a pilot study using calcein staining as a method to elucidate this question was also conducted.

2 Material and methods

Local broodstock were collected in January 2012 from the outer Hardangerfjorden, located on the south-western coast of Norway. Ten 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. *Pecten maximus* is a simultaneous hermaphrodite, and some sperm will remain in the genital tract, resulting in self-fertilization (Mackie, 1984). Self-fertilization rate was checked before cross-fertilization, and egg batches with less than 10 % self-fertilization were used in the experiment. Eggs from one individual were cross-fertilized with sperm from three other individuals. Egg : sperm ratio was

Table 1. Measured and calculated water parameters given as mean ± sd for four different pCO_2 groups (µatm). Carbon chemistry values were computed based on daily measurements (0–6 days) of pH_{NBS} in all replicates ($n = 4$, $^*n = 3$), means of hourly temperature measurements in three tanks ($n = 468$), salinity (daily), and total alkalinity (11 analyses from 3 dates) in seawater running into the lab. Coefficient of variation (CoV) range (sd as % of mean) is shown for variation in CO_2 (ppm) between days (d) for the replicates, and between replicates (r) for all days.

pCO_2 group	469 µatm	807 µatm	1164 µatm*	1599 µatm
Measured parameters				
pH_{NBS}	7.98 ± 0.01	7.78 ± 0.05	7.64 ± 0.07	7.51 ± 0.10
Salinity	35.0	35.0	35.0	35.0
Temperature (°C)	15.6 ± 0.4	15.6 ± 0.4	15.7 ± 0.3	15.6 ± 0.4
A_T (mmol kg^{-1} SW)	2341 ± 11	2341 ± 11	2341 ± 11	2341 ± 11
Calculated parameters				
pCO_2 (µatm)	469 ± 9	807 ± 106	1164 ± 230	1599 ± 421
HCO_3^- (µmol kg^{-1} SW)	1973 ± 5	2097 ± 24	2159 ± 25	2200 ± 28
CO_3^{2-} (µmol kg^{-1} SW)	149 ± 2	99 ± 10	74 ± 10	57 ± 11
CO_2 (µmol kg^{-1} SW)	17.2 ± 0.3	29.5 ± 3.8	42.5 ± 8.1	58.4 ± 15.1
$\Omega_{aragonite}$	2.28 ± 0.03	1.52 ± 0.15	1.13 ± 0.15	0.88 ± 0.17
CO_2 (ppm)	477 ± 9	821 ± 108	1184 ± 222	1627 ± 429
CoV (d) CO_2	1.14–2.2	12.5–15.9	18.9–20.6	23.3–33.7
CoV (r) CO_2	0.0–2.2	1.3–11.4	1.4–11.4	1.2–13.8

approximately 1 : 100. Fertilized egg batches were mixed and incubated 1–2 h after fertilization. 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 an ambient pH_{NBS} of 7.98 (control) and mean pH_{NBS} levels of 7.71, 7.64, and 7.51, corresponding to a pCO_2 level of 469, 807, 1164, and 1599 µatm (Table 1). The pH levels were used based on the predicted drop of 0.5 units from present day to year 2250 (IPCC, 2001). Mean temperature based on hourly measurements in three replicates (± sd) was 15.6 ± 0.3 °C ($n = 495$). Seawater with a salinity of 35 ppm was pumped from 160 m depth and filtered through a sand filter before temperature was adjusted in a heat pump. The water was aerated and finally filtered through a 50 µm filter. The experiment was conducted in a flow-to-waste system consisting of circular fiberglass tanks with slightly conical bottom, resulting in a depth difference of 22 mm between tank centre 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. One replicate was lost after three days in the group exposed to a mean pH_{NBS} of 7.64 (1184 ppm) due to overflow. To prevent escape of embryos, water flow in the experimental tanks was turned off overnight at the incubation of 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 of pressurized air 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_{NBS} 5.80, made from mixing CO_2 gas and seawater with an ambient pH_{NBS} of 7.95. The pH_{NBS} in each mixing tank was continuously adjusted to preset levels by addition of stock solution with dosage pumps (IWAKI) controlled by feedback from pH electrodes to pH transmitters (Endress & 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 the 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 equipped with a Mettler Toledo InLab® ExpertPro pH-probe, calibrated with 4.00 and 7.00 buffers (Certipur® buffer solutions, Merck KGaA, 64271 Darmstadt, Germany) traceable to standard reference material from NIST (NBS). Total alkalinity (A_T) was analysed in four replicate samples on 9, 15, and 26 March 2012 by a TitraLab radiometer. Salinity was checked daily using 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 values (µatm) corresponding to the pH_{NBS} values were calculated from the daily average temperature (°C) based on hourly recordings, salinity, A_T and pH using the macros in Ernie Lewis' "CO2SYS.BAS" basic program (Pierrot et al., 2006).

Larvae samples were taken 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 400 mL. On day 2 a volume of 800 mL from each replicate was withdrawn to measure shell size and classify larval deformities. When terminating the experiment after seven days, all tanks were drained and a total sample from each replicate was collected and preserved to determine final shell size, survival, and classify deformities. To determine timing of shell calcification, larvae from ambient pH in an additional tank were stained for 18–20 h from day 1 to day 2.

When collected for preservation, embryos and larvae were retained on a 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 was investigated. The formalin was made from mixing 8.14 g of disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$, Merck 1.06580.1000) and 4.00 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, Merck 1.06346.1000) with 100 mL of 37 % formalin in 900 mL of distilled water.

A calcein stock solution was made from mixing 1 g of calcein (Sigma C0875-5G) in 1 L of distilled water adjusted to pH_{NBS} 7.0 using a sodium bicarbonate ($NaHCO_3$, Merck 1.06329.1000) solution of 2.4 g in 400 mL of distilled water (Moran, 2000; Moran and Marko, 2005; Fitzpatrick et al., 2010). For staining of calcium, 20 mL of calcein stock solution was added to 400 mL of seawater with larvae, giving a final calcein concentration of 22 mg L^{-1}. Only larvae at ambient pH_{NBS} were stained, as the aim of this pilot study was to elucidate when the calcification of the larvae shell is normally initiated. We assumed that the pH was not affected during staining since the calcein stock solution was less than 5 % of the total water volume; however the pH was not measured. Calcein has little or no effect on mollusc larvae survival or growth (Moran, 2000; Moran and Marko, 2005; Chick, 2010; Fitzpatrick et al., 2010).

Live (calcein stained) and preserved 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 4.8 µm from printed photographs. Survival was based on the initial number of fertilized eggs, and was estimated by concentrating all day-7 larvae in 25–110 mL and counting 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. The number of trochophores classified on day 2 was 76–150, and 46–178 veligers were examined on day 7. Since we were unable to find any published study on deformities in scallop larvae, deformities were categorized according to deformities as described in larvae of mussel (*Mytilus* sp) and the Pacific oyster (*C. gigas*) (His et al., 1997). We observed the same "convex hinge" and "indented shell margins" in our larvae, but called the categories "hinge deformity" and "edge deformity", respectively. We classified preserved larvae into four categories according to shell shape, and counted (1) normal (Fig. 1a), (2) hinge deformity (Fig. 1b–c), (3) edge deformity (Fig. 1d–e), and (4) both edge and hinge deformity (Fig. 1f). Trochophore larvae on day 2 were only classified into category 1 and 2, since shell edge was not visible until the shell valves covered the whole larva. In veligers on day 7, all four categories were used.

Live, calcein-stained larvae were photographed at 10×40 magnification using an U-MSWB2 cube filter (excitation 420–440 nm, emission 475 nm) 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.) was used to run statistical tests. Arcsine-transformed survival data and shell dimension data were not confirmed to normality (Shapiro–Wilk's W test), and significant effects were tested using Kruskal–Wallis ANOVA by ranks. Differences between groups were tested using multiple comparison p values (two-tailed). The rest of the data were tested using one-way ANOVA, followed by unequal N HSD post hoc test to find differences between groups. The significance level used in all tests was set to 0.05.

3 Results

3.1 Initial variation in pCO_2 (µatm)

Measured and calculated water parameters for the four pCO_2 groups are given in Table 1. Salinity and total alkalinity (A_T) showed little or no variation, including in periods before and after the experiment (February–May). The pCO_2 values (µatm) for the elevated groups showed a sharp decrease during the first night after incubation due to a discontinuation in seawater flow in the exposure tanks (Fig. 2). This drop in pCO_2 (delta, ΔpCO_2) increased with increasing pCO_2 levels, and fell from a mean of 2489 to 1203 µatm (ΔpCO_2 of 1286 µatm) in the highest pCO_2 group. The ambient group showed only an insignificant ΔpCO_2 of 21 µatm. Naturally, the variation in pCO_2 values given as the coefficient of variation (CoV), increased with increasing pCO_2 levels, and was higher between days for replicates than between replicates on any day (Table 1).

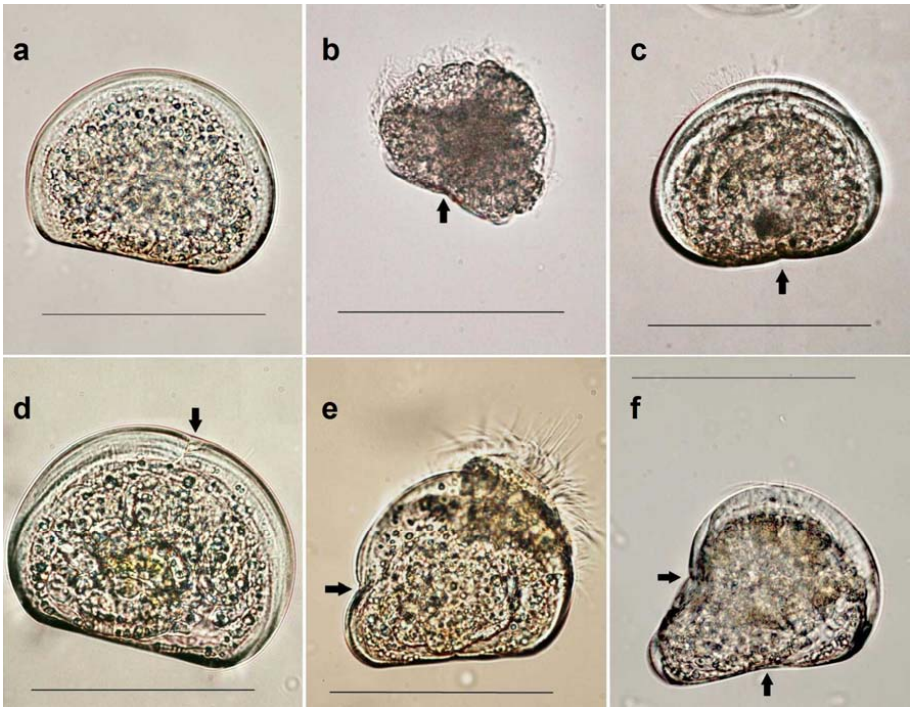

Fig. 1. Shell shape categories observed in *Pecten maximus* larvae. **(a)** Normal shell shape in formalin-preserved day-7 veliger larva; **(b)** hinge deformity in formalin-preserved day-2 trochophore larva and **(c)** in day-3 veliger larva; **(d)** edge deformity in shell edge centre of a live day-7 veliger larva and **(e)** in shell edge side of a live day-6 veliger larva; **(f)** both hinge and edge deformity in a live day-7 veliger larva. The scale bar is 100 µm.

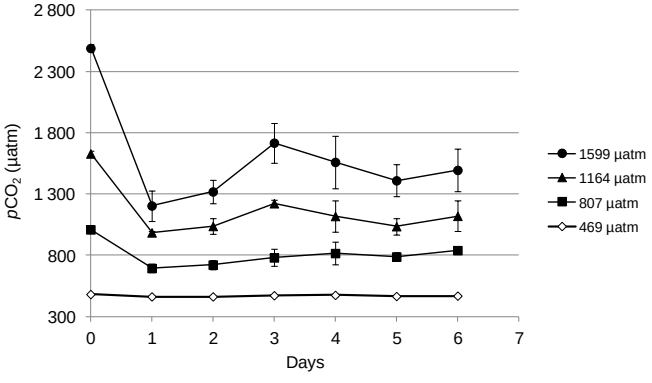

Fig. 2. The daily means of pCO_2 (µatm) ± sd for replicates in the treatments and ambient group during the experimental period of seven days ($n = 4$, $n = 3$ for 1164 µatm).

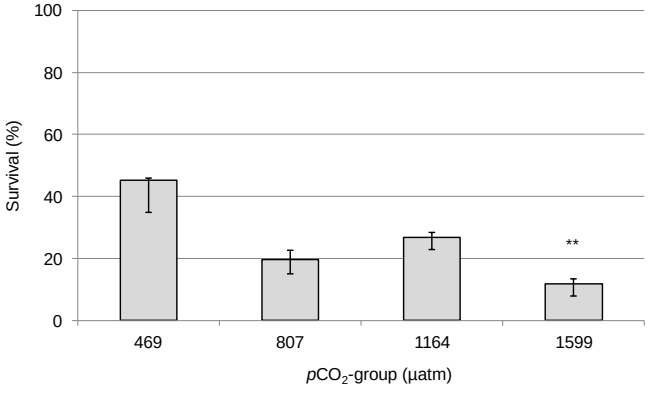

Fig. 3. Survival of day-7 scallop larvae given as median ± quartile in the four pCO_2 groups as percentage of eggs incubated. ** Significantly different from the other pCO_2 groups.

3.2 Survival

The median survival of day-7 veligers based on the initial number of incubated eggs reared at ambient pCO_2 was 45 % (range 25–46) and decreased with elevated pCO_2 levels (Fig. 3). At the highest pCO_2 level the median survival was 12 % (range 6–13 %). There was a significant effect of pCO_2 on survival ($p = 0.013$), but only the group at the highest pCO_2 level was significantly different from the control.

3.3 Shell size

Shell length and height on day 7 were both negatively affected by an increase in pCO_2 level ($p < 0.001$ for both) showing a decrease in median values of 8 and 15 %, respectively, when pCO_2 increased from 469 µatm (ambient) to the highest pCO_2 of 1599 µatm (Fig. 4). The median shell length and height were 115 and 91 µm, respectively, in the ambient

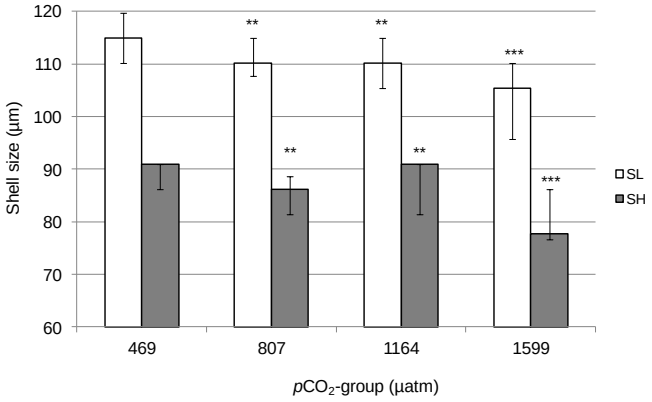

Fig. 4. Scallop larvae shell length (SL) and height (SH) on day 7 given as median ± quartile for four pCO_2 groups. Each median represents measurements of 21–60 individuals in 3–4 replicates. Unmarked, **-, and ***-marked CO_2 groups are significantly different within the same variable.

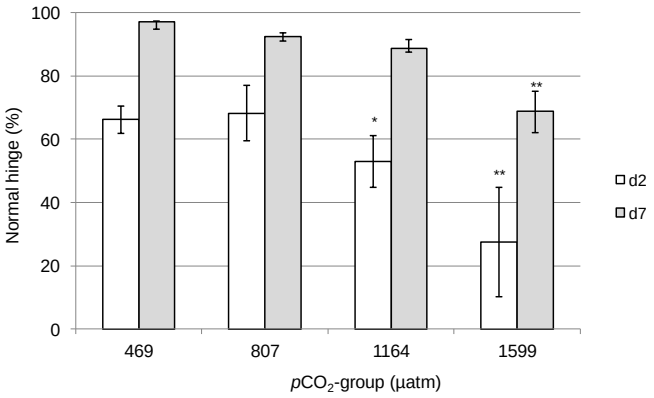

Fig. 5. Percentage of larvae showing a normal, straight hinge on day 2 (d2) and day 7 (d7) based on the total larval number. Values are means ± sd for d2, and medians ± quartiles for d7 (n =4, and $n = 3$ for 1164 µatm). * denotes not significantly different from any group within same variable; **, significantly different from unmarked groups within same variable.

group. All the pCO_2 groups were significantly different for both parameters, except for the two pCO_2 groups 807 and 1164 µatm.

3.4 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. Around 28–68 % of all individuals in the different pCO_2 groups on day 2 had developed a normal-looking straight hinge (Fig. 5). On day 7 the percentage of normal hinges was higher: 69–97 % for all pCO_2 groups. The percentage of larvae with a normal hinge was negatively affected by pCO_2 on both days, ($p = 0.004$ and $p = 0.021$ for d2 and d7, respectively), and the lowest percentages were found at the highest pCO_2 level (Fig. 5). The percentages of all four shell shape categories on day 7 were affected by increasing pCO_2 level ($p < 0.000$–0.005) (Fig. 6). The percentage of normally shaped larval shells decreased from 64 to 28 % with an increase in pCO_2 level from ambient to 1599 µatm. The effect of elevated pCO_2 level seemed to follow no clear pattern as the ambient group was significantly different only from the least (807 µatm) and the most elevated pCO_2 level (1599 µatm). Shell edge deformities accounted for 30–57 % in the different pCO_2 groups (Fig. 6), and the control was significantly different only from the least elevated group. The percentage of larvae with hinge deformities, and both hinge and edge deformities, increased when the pCO_2 level increased. Only the most elevated pCO_2 group was significantly different from the ambient group for these two shell deformity categories. Variation within replicates was high, and the coefficient of variation (CoV) ranged between 5 and 70 % for all pCO_2 groups and categories.

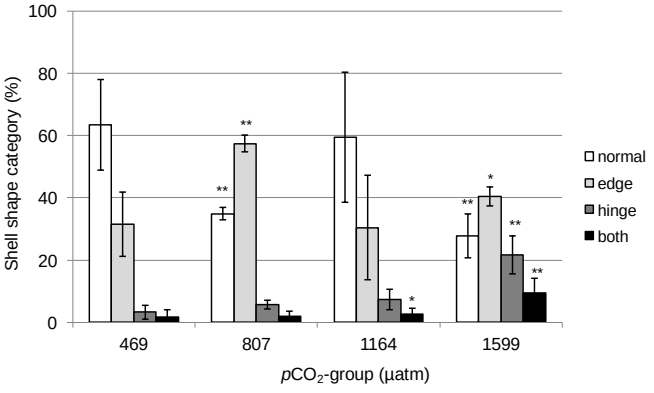

Fig. 6. Percentage of the four scallop larvae shell shape categories on day 7 for different pCO_2 groups. Values are mean ($n = 4$, $n = 3$ for 1164 µatm), and error bars are sd. * denotes not significantly different from any group within same category; **, significantly different from unmarked groups within same category.

3.5 Calcification

Trochophore larvae in ambient seawater (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 organic shell started between day 1 and day 2.

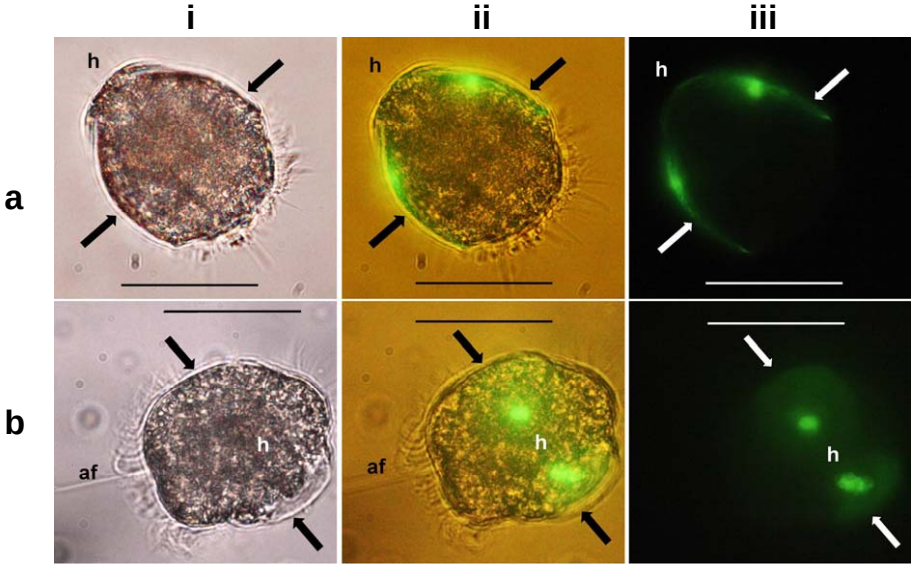

Fig. 7. Stereomicroscopic images of live trochophore scallop larvae stained with calcein from day 1 and overnight (18–20 h). **(a)** A side view with the hinge area (h) oriented towards the upper-left corner and a newly formed shell valve on each side (arrows). **(b)** The hinge area (h) and early shell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

4 Discussion

4.1 Non-feeding regime

In the present study, scallop larvae were not fed during the 7-day experimental period. Lack of food is known to affect the energy status of scallop larvae after only three days (Andersen et al., 2012); however, the survival and growth in the control group indicate that the unfed larvae were healthy and viable. Previous OA experiments on bivalve larvae support that the decreased survival, smaller size, and increased percentage of deformed *P. maximus* larvae found in the highest pCO_2 group was caused by the change in water chemistry rather than the feeding regime (feeding or non-feeding) (Table 2).

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. 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. We believe that in nature, bivalve larvae may experience shorter or longer periods of food scarcity; thus unfed larvae may not be uncommon, and most species may be quite well adapted to such conditions. More studies are needed to elucidate how well *P. maximus* larvae are able to handle the future pCO_2 levels at different feeding regimes.

4.2 Initial drop in pCO_2 level

The initial drop in pCO_2 level was most likely due to both the discontinuation in seawater flow and gas exchange at the water surface due to slow aeration during the first 12 h. This drop may have added additional physiological stress to the scallop larvae, especially in the higher pCO_2 groups. Both the delta pCO_2 and the initial maximum values may have contributed to an overestimation of the effect on all parameters (survival, growth, and deformities) that were given for the average pCO_2 levels, especially for the two groups exposed to the highest levels. Surface waters (upper 100 m) of the open ocean vary between pH 7.9 and 8.3 depending on season and geographic region, and coastal areas may even show higher fluctuations and relatively rapid changes in pCO_2 levels may be expected (Raven et al., 2005; Blackford and Gilbert, 2007; Kerrison et al., 2011, and references therein). *P. maximus* is most abundant at 5–50 m depth in Norwegian coastal waters, and may be exposed to such variable levels in pH in nature. However, short-term fluctuations within hours have not yet been quantified to our knowledge. Comparing our results with OA studies using more or less the same pCO_2 or pH levels indicates that the effects we measured were most likely due to elevated pCO_2 over time rather than a result of the sudden drop in the first hours of the experiment (Talmage and Gobler, 2010, 2009; Watson et al., 2009). Experiments with rapid changes in pH can elucidate how variation experienced under natural conditions may affect marine organisms. However, such experiments should be conducted under controlled conditions, not as a result of methodological constraints.

Table 2. Overview of ocean acidification (OA) effect studies. The focus is on bivalves, but some studies on other calcifying invertebrates are included. "Volume" is the experimental unit volume, "n" is the number of replicates, "water exchange" is the flow rate in the flow-through system, and if the system is stagnant "water exchange" gives the exchange rate of the total volume. NG – information not given in the original publication.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
Bivalves										
Argopecten irradians bay scallop	Embryos (0 d)	1 L (3, 4)	350 (350)	Stagnant (2 times a week, + antibiotics)	24, 28	3 weeks	Yes (1)	pH_{NBS} 8.2, 8.1, 7.8 CO_2 220–247, 374–387, 756–795 ppm	Development (–) Survival (−) Growth (–) Lipid synthesis (–) for both OA and temperature, additive effects	Talmage and Gobler (2011)
A. irradians	Larvae (1 d)	1 L (4)	200 (200)	Stagnant (2 times a week, + antibiotics)	24 (28)	52 days	Yes (1)	CO_2 244, 387, 739, 1529 ppm, pH_T 8.17, 8.041, 7.801, 7.530	at pH < ambient Growth (–) Metamorphosis (–) Survival (–) Lipid accumulation rates (–) Normal shell development (–) Shell thickness (–) at pH >ambient Growth (+) Metamorphosis (+) Survival (+) Lipid accumulation rates (+) Shell thickness (+)	Talmage and Gobler (2010)
A. irradians	Larvae (1 d)	1 L (4)	100 (100)	Stagnant (2 times a week, + antibiotics)	24 (28)	18–20 days	Yes (1)	pH_T 8.1, 7.8, 7.5 pCO_2 39, 66, 152 Pa	Survival (−) Metamorphosis (−) Size (−)	Talmage and Gobler (2009)
A. irradians	Juveniles (15.9 mm)	10 L (3)	10 (1)	Stagnant (every 3 days, + antibiotics)	24, 28	45 days	Yes (1)	pH_{NBS} 8.1, 7.6 CO_2 400–400, 1665–1737 ppm	OA Shell growth (−) Tissue growth (0) Temperature Shell growth (−) Tissue growth (−) no additive effects	Talmage and Gobler (2011)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
Pecten maximus (king scallop)	Adults (108.5 mm shell height)	200 L (8)	10 (0.05)	Recirculation (exchanged twice a week)	4, 10	33–60 days	Yes (3)	pCO_2 at 4 °C: 0.040 and 0.110 kPa pCO_2 at 10 °C: 0.040 and 0.115 kPa pH_{NBS} at 4 °C: 8.19 and 7.76 pH_{NBS} at 10 °C: 8.25 and 7.81 Temperature	Temperature Survival (−) OA Clapping performance (−) Metabolic rate (−)	Schalkhausser et al. (2012)
Placopecten magellanicus (sea scallop)	Gametes, oocytes, embryos	NG	NG	NG	10 (25)	180 h	No	pH 7.0, 7.5, 8.0, 8.5	Extrusion of polar bodies (0) Duration of first miotic division shorter at pH 8–8.5 Completion of first embryotic cleavage (−) at pH 7.0 and 7.5 Levels of polyspermy (+) at pH 7.0 and 7.5	Desrosiers et al. (1996)
Chlamys nobilis noble scallop	Juveniles (40–44 mm)	10 L (5)	4 (0.4)	Stagnant (daily)	25 (32)	5 days	Yes (1)	pH_{NBS} 8.1, 7.7, 7.4	Clearance (−) Respiration rate (0), (−) at pH 7.4 Excretion (−) at pH 7.4	Liu and He (2012)
Crassostrea gigas Pacific oyster	Gametes	100 mL (3)	500 (5000)	Stagnant	18, 22, 26, 30 (35)	48 h	No	pCO_2 375, 600, 750, 1000 µatm	D-veliger % (−) Abnormality % (+) Size (−) All less at optimum temperatures, more when fertilized at elevated pCO_2	Parker et al. (2010)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
C. gigas	Embryo (0 d)	4.5 L (3)	67 500 (15 000)	Stagnant	18.9 (34.0)	3 days	No	pH_T 8.03, 7.72, 7.41, 7.67, 7.62 pCO_2 449, 1020, 2171, 494, 3730 A_T 2453, 2446, 2443, 1094, 6727 Ω_{ar} 2.8, 1.5, 0.8, 1.6, 3.5	Above CO_3^{2-} saturation level: Developmental success (0) Shell length (0) Shell area (0) Incorp. calcium (0) Below CO_3^{2-} saturation level: Developmental success (−) Shell length (−) Shell area (−) Incorp. calcium (−)	Gazeau et al. (2011)
C. gigas	Embryo (0 d)	50 mL (5)	< 500 (< 10 000)	Stagnant (sealed)	23 (33.7)	2 days	No	pH_{NBS} 8.21, 7.42	Normal development of gastrula (0) Shell size (−) Normal shell shape (−) Shell mineralization (−)	Kurihara et al. (2007)
C. gigas	Larvae (0 d)	20 L (3)	60 000	Flow	17.9 (30.6)	148 h	Yes (1)	pH_{TS} 8.09, 7.76, 7.37 pCO_2 580, 1386, 3573 µatm	Fertilization (−) Sperm motility and velocity (−) Survival (−) Normal shell development (−) Growth (−)	Barros et al. (2013)
C. gigas	Umbonate (137 µm)	100 mL	250 (2500)	Stagnant (daily)	18, 22, 26, 30 (35)	4 days	Yes (3)	pCO_2 375, 600, 750, 1000 µatm Temperature	Size (−), not at 18 °C	Parker et al. (2010)
C. gigas	Pediveliger (246 µm)	100 mL	200 (2000)	Stagnant (daily)	18, 22, 26, 30 (35)	4 days	Yes (3)	pCO_2 375, 600, 750, 1000 µatm Temperature	Size (−), not at 30 °C	Parker et al. (2010)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
C. gigas	Juveniles (5.5 mm)	100 mL	15 (150)	Stagnant (daily)	18, 22, 26, 30 (35)	4 days	Yes (3)	pCO_2 375, 600, 750, 1000 µatm Temperature	Size (−), more so at 30 °C	Parker et al. (2010)
C. virginica eastern oyster	Embryo	5 L (2)	5 (1)	Stagnant (every other day)	21 (30)	2 weeks	Yes	380, 3500 µatm	Standard metabolic rate (+)	Beniash et al. (2010)
C. virginica	Embryos (0 d)	1 L (3, 4)	350 (350)	Stagnant (2 times a week, + antibiotics)	24, 28	3 weeks	Yes (1)	pH_{NBS} 8.2, 8.1, 7.8 CO_2 220–247, 374–387, 756–795 ppm	Development (−) Survival (−) Growth (−) Lipid synthesis (−) for both OA and temperature, additive effects	Talmage and Gobler (2011)
C. virginica,	Larvae (1 d)	1 L (4)	100 (100)	Stagnant (2 times a week, + antibiotics)	24 (28)	18–20 days	Yes (1)	pH_T 8.1, 7.8, 7.5 pCO_2 39, 66, 152 Pa	Survival (−) Metamorphosis (−) Size (−) only at 152 Pa	Talmage and Gobler (2009)
C. virginica	Juveniles (< 1 mm)	5 L (2)	50 (10)	Stagnant (every other day)	21 (30)	20 weeks	Yes (3)	pCO_2 380, 3500 µatm	Survival (−) Dry shell mass (−) Soft-tissue mass Shell fracture toughness (−) Shell growth (−) Shell area (0) Standard metabolic rate (+)	Beniash et al. (2010)
C. virginica	Juveniles (7 weeks)	30 L ($6_{control}$, 11–12)	NG	Stagnant (every other day)	21 (30, 15 psu)	11 weeks	Yes (3)	400, 700–800 µatm Salinity	Survival (−) Tissue energy stores (−) Soft tissue growth (−) salinity more than high CO_2	Dickinson et al. (2012)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
C. virginica	Juveniles (11.5 mm)	10 L (3)	10 (1)	Stagnant (every 3 days, + antibiotics)	24, 28	45 days	Yes (1)	pH_{NBS} 8.1, 7.6 CO_2 400–400, 1665–1737 ppm Temperature	OA Shell growth (−) Tissue growth (0) Temperature Shell growth (−) (0) at pH 7.6 Tissue growth (0) *C. virginica* No additive effects	Talmage and Gobler (2011)
Mercenaria mercenaria quahog (hard clam)	Embryos (0 d)	1 L (3, 4)	350 (350)	Stagnant (2 times a week, + antibiotics)	24, 28	3 weeks	Yes (1)	pH_{NBS} 8.2, 8.1, 7.8 CO_2 220–247, 374–387, 756–795 ppm Temperature	Both OA and temperature Development (−) Survival (−) Growth (−) Lipid synthesis (−) additive effects	Talmage and Gobler (2011)
M. mercenaria	Larvae (1 d)	1 L (4)	100 (100)	Stagnant (2 times a week, + antibiotics)	24 (28)	18–20 days	Yes (1)	pH_T 8.1, 7.8, 7.5 pCO_2 39, 66, 152 Pa	Survival (−) Metamorphosis (−) Size (−)	Talmage and Gobler (2009)
M. mercenaria	Larvae (1 d)	1 L (4)	100 (100)	Stagnant (2 times a week, + antibiotics)	24 (28)	18–20 days	Yes (1)	pH_T 8.02, 7.97, 7.88, 7.79 pCO_2 41, 49, 60, 73 Pa	Survival (−) Metamorphosis (−)	Talmage and Gobler (2009)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
M. mercenaria	Larvae (1 d)	1 L (4)	200 (200)	Stagnant (2 times a week, + antibiotics)	24 (28)	36 days	Yes (1)	CO_2 247, 380, 742, 1529 ppm, pH_T 8.171, 8.052, 7.801, 7.532	pH < ambient Growth (−) Metamorphosis (−) Survival (−) Lipid accumulation rates (−) Normal shell development (−) Shell thickness (−) pH >ambient Growth (+) Metamorphosis (+) Survival (+) Lipid accumulation rates (+) Shell thickness (+)	Talmage and Gobler (2010)
M. mercenaria	Juveniles (6.1 mm)	10 L (3)	10 (1)	Stagnant (every 3 days, + antibiotics)	24, 28	45 days	Yes (1)	pH_{NBS} 8.1, 7.6 CO_2 400–400, 1665–1737 ppm	OA Shell growth (0) Tissue growth (0) Temperature Shell growth (−) Tissue growth (0) No additive effects	Talmage and Gobler (2011)
Mercenaria spp.	Post-larvae – juveniles (0.39–2.9 mm)	50 mL (1, 3, 4)	1–2 g (20–40 g)	Stagnant (daily)	20	8 h	Yes (1+ Shellfish diet)	pH_{NBS} 8.02, 7.64, 7.41 pCO_2 424, 1120, 1950 µatm	Calcification rate (−), all sizes, smaller sizes more affected, and at higher pH Dependent on hatchery populations (hybridized and pure *Mercenaria*)	Waldbusser et al. (2010)
Mytilus californianus California mussel	Larvae	2 L (6)	1000 (500)	Stagnant (every 2 days)	15	9 days	Yes (1)	380, 540, 970 ppm	Shell length (−) Shell thickness (−) Shell size (−) Tissue mass (−)	Gaylord et al. (2011)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
Mytilus edulis blue mussel	Embryo (0 d)	130 L (3)	(10 000)	Stagnant	16.2–16.7 (31.9–32.1)	2 days	No	pH_{NBS} 8.1 (460–640 µatm), pH 7.8 (1100–1200 µatm), pH 7.6 (460–640 µatm)	Shell length (−) Shell thickness (−) Hatching rate (0)	Gazeau et al. (2010)
M. edulis	Larvae (2 d)	130 L (3)	(10 000)	Flow	19.2,19.5 (31.4, 31.5)	13 days	Yes (2–3)	pH_{NBS} 8.1, 7.8	Shell length and thickness (−) Survival (0)	Gazeau et al. (2010)
M. edulis (20 % hybrid with *Mytilus trossulus*)	Post-larvae	500 mL (7)	100 (200)	Stagnant (400 mL daily)	17	7 weeks	Yes (1)	pCO_2 470, 1020, 2110, 3350 µatm 3 food levels	OA Shell size (−) Survival (0) Calcification (−) Organic growth (0) Food Shell size (+) Survival (0) Calcification (+) Organic growth (+) Explained most of the variation	Thomsen et al. (2013)
M. edulis	Juveniles – Adults (5.5, 13.3 mm)	16 L (4)		Flow (100 mL min^{-1})	13.8 (15)	8 weeks	Yes (1)	pH_{NBS} 8.13, 7.72, 7.26 pCO_2 493, 1332, 3898 µatm	Shell length (−) at 3898 µatm Individual aragonite platelet thickness (−) at 3898 µatm Aragonite layer thickness (0) Calcite layer thickness (0) Shell dissolution Index (+) Shell dissolution area (+)	Thomsen et al. (2010)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
M. edulis	Juveniles (16.6–17.6 mm)	18 L (4)	4 (0.22)	Flow (50 mL min^{-1})	4.7–5.4 (16)	7 weeks	Yes (1)	pCO_2 mean 54, 117, 248, 385 Pa pH_{NBS} mean 8.05, 7.73, 7.43, 7.19 Food 0.6 and 5.7 cells µL^{-1}	OA Shell growth (−) at 385 Pa Internal shell dissolution (−) no interactions Food Shell growth (+) Inernal shell dissolution (+) interactions?	Melzner et al. (2011)
M. edulis	Juveniles (45–55 mm)	50 L (2)	32 (0.64)	Flow (60 mL min^{-1})	16.03–16.11 (35.13–35.37)	60 days	Yes (1)	pH_{NBS} 8.0, 7.8, 7.6, 6.5	Health (NRR assay) (−)	Beesley et al. (2008)
M. edulis	Adults (76 mm)	16 L (2?)	6 (0.4)	Flow	12.5 (11.8)	14 days	Yes (3)	pH_{NBS} 8.05, 7.89, 7.81, 7.70, 7.56, 7.08 pCO_2 464, 661, 789, 1046, 1480, 4254 µatm	pH_e(−) Haemolymph Mg^{2+} (0) Haemolymph Ca^{2+} (0)	Thomsen et al. (2010)
M. galloprovincialis Mediterranean mussel	Embryos	50 mL (6)	400 (< 10 000)	Stagnant (55, 96 and 121 h)	13.0 (35.5)	6 days	No	pH_{NBS} 8.13, 7.42	Development rate from trochophores (−) Shell size (−) Normal shell shape (−)	Kurihara et al. (2008)
Mytilus trossulus	Larvae	22 mL (240)	72.6 (3300)	Stagnant	12 (34.3)	60 h	No	pCO_2 253, 571 pH_{NSB} 8.3, 7.9	Shell size (−)	Sunday et al. (2011)
Perna viridis green-lipped mussel	Juveniles (40–44 mm)	10 L (5)	4 (0.4)	Stagnant (daily)	25 (32)	5 days	Yes (1)	pH_{NSB} 8.1, 7.7, 7.4	Clearance (−) Respiration rate (0) Excretion (−) at pH 7.4	Liu and He (2012)
Pinctada fucata pearl oyster	Juveniles (40–44 mm)	10 L (5)	4 (0.4)	Stagnant (daily)	25 (32)	5 days	Yes (1)	pH_{NSB} 8.1, 7.7, 7.4	Clearance (−) at pH 7.4 Respiration rate (0) Excretion (−)	Liu and He (2012)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
Ruditapes decussatus grooved carpet shell (clam)	Juveniles (10.2 mm)	15 L (3)	100 (6.7)	Flow (3–4 times day^{-1})	22–23 (31–32)	75 days	Yes (2)	pH 8.25, 7.85, 7.67	Net calcification (0) Size (0) Weight (0) may be due to natural elevated total alkalinity Survival (+) may be due to spawning at pH 8.25, 7.85	Range et al. (2011)
Saccostrea glomerata Sydney rock oyster	Gametes	100 mL (3)	500 (5000)	Stagnant	18, 22, 26, 30 (35)	48 h	No	pCO_2 375, 600, 750, 1000 µatm Temperature Fertilization	D-veliger % (−) Abnormality % (+) Size (−) All less at optimum temperatures, more when fertilized at elevated pCO_2	Parker et al. (2010)
S. glomerata	Gametes	200 L (3)	3 million (15 000) After 12 h: 1 million (5000)	Stagnant (every 2 days)	24 (34.6)	19 days	Yes (3)	pH_{NSB} 8.2, 7.9 pCO_2 380, 856 µatm Parental OA exposure, and origin (wild and selected)	Survival (−), also (−) for wild vs. breed parental origin, no interaction with OA, (0) for parental exposure Development rate to veliger (−), (+) for parental exposure, and (+) for bred origin Development rate to eyed (−), interaction parental exposure – origin – OA Shell length (−), (0) for bred and exposed parents, (+) for exposed parents	Parker et al. (2012)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
S. glomerata	D-veligers (24 h)	60 L (1)	90 000 (1500)	Flow	26	8 days	Yes (2)	pH: 8.1, 7.8, 7.6 pCO_2 580, 1386, 3573	Survival (−) Growth (−) Shell morphology (−) Amount of empty shells (−) Shell surface characteristics (−) Larval dry mass (0)	Watson et al. (2009)
S. glomerata	Umbonate (142 µm)	100 mL	250 (2500)	Stagnant (daily)	18, 22, 26, 30 (35)	4 days	Yes (3)	pCO_2 375, 600, 750, 1000 µatm Temperature	Size (−), not at 18 °C	Parker et al. (2010)
S. glomerata	Pediveliger (264 µm)	100 mL	200 (2000)	Stagnant (daily)	18, 22, 26, 30 (35)	4 days	Yes (3)	pCO_2 375, 600, 750, 1000 µatm Temperature	Size (−), not at 18 and 26 °C	Parker et al. (2010)
S. glomerata	Juveniles (2.0 mm)	100 mL	15 (150)	Stagnant (daily)	18, 22, 26, 30 (35)	4 days	Yes (3)	pCO_2 375, 600, 750, 1000 µatm Temperature	Shell growth (−), more so at 18 and 22 °C	Parker et al. (2010)
S. glomerata	Post-larvae	120 mL (3)	30 (250)	Stagnant (daily)	25 (35)	4 days	Yes (3)	pCO_2 375, 1000 ppm* Wild and mass-selected populations (*msp*), and families within *msp*	Shell growth (−), *msp* were less affected, large variations between families	Parker et al. (2011)
spp from bivalvia	NG	38 L (6)	NG	Stagnant (filtration 600 L h^{-1}, 75 % every 14 days)	25	60 days	Yes (several)	pCO_2 409, 606, 903, 2856 pH_{NBS} 8.03, 7.85, 7.72, 7.31	Net calcification (−) for scallops, oysters, hard clams, soft clams	Ries et al. (2009)

Table 2. Continued.

Species	Life stage (age/size)	Volume (n)	Stocking density (ind L^{-1})	Flow or stagnant (water exchange)	Temp. °C (Salinity)	Duration	Food supply (spp)	OA Treatment	Effects: Negative (−) None (0) Positive (+)	Reference
Others										
Dendraster excentricus sand dollars (sea urchin)	Gastrulae	3.7 L (3)	7400 (2000)	Stagnant (every other day)	20		Yes (2)	CO_2 380, 1000 ppm (pH_{NBS} 8.06, 7.75)	Stomach size (−) Body size (−) Swimming speed (0) between-family variation	Chan et al. (2011)
Echinometra viridis tropical urchin	Juveniles NG (dw 1.2–6.1 g)	34 L	6–8	Stagnant (filtered at 757 L h^{-1})	20.4, 29.9 (32)	60 days	Yes (NG)	CO_2 524, 827 ppm at 20.4 °C CO_2 448, 783 ppm at 30.0 °C	Survival (0) Calcification rate (−), (+) by temperature	Courtney et al. (2013)
Madracis auretenra coral	Branches	250 mL (3)	4*	Stagnant	28 (36)	2 h	No	pH_T 8.06, 7.78, 7.60, CO_3^{-2} 105, 150, 260 µmol kg^{-1}	Calcification (−) at pH 8.06 if HCO_3^- < 1800 1M;(0) or (+) at pH 7.6–7.8 if HCO_3^- >1800 1M	Jury et al. (2010)
Tripneustes gratilla sea urchin	Embryo (0 d)	100 mL (3)	100–200 (1000–2000)	Flow (7.8 mL min^{-1})	24, 27, 30	5 days	No	pH_{NBS} 8.15, 7.85, 7.65 Temperature	Growth (−) Calcification (−)	Brennand et al. (2010)
spp from: Crustacea Cnidaria Echinoidea Rhodophyta Chlorophyta Gastropoda Annelida	NG	38 L (6)	NG	Stagnant (filtration 600 L h^{-1}, 75 % every 14 days)	25	60 days	Yes (several)	pCO_2 409, 606, 903, 2856 pH_{NBS} 8.03, 7.85, 7.72, 7.31	Net calcification (−) for: corals, urchins, whelks, serpulid worms, periwinkles (+) for: limpets, purple urchins, coralline red algae, calcareous green algae	Ries et al. (2009)
Strongylocentrotus fransiscanus	Larvae	22 mL (1000)	39.6 (1800)	Stagnant	12 (34.3)	7 days	No	pCO_2 253, 571 pH_{NSB} 8.3, 7.9	Larvae size (−)	Sunday et al. (2011)

4.3 Inter- and intra-species response and synergistic factors

In the present study scallop larvae showed a reduction in shell size, survival, and percentage of normally developed larvae from the ambient group to the highest pCO_2 group (discussed in further detail in the sections below). These responses to OA seem to vary little between bivalve species (Table 2). However, the magnitude of the response vary, although the pH window, temperature, and rearing volume used during experiments vary, and one should be careful in drawing conclusions about species-specific effects between studies. Comparative studies of two (Sunday et al., 2011; Parker et al., 2010; Talmage and Gobler, 2010) or several species (Liu and He, 2012; Ries et al., 2009; Talmage and Gobler, 2009, 2011) mostly conclude that species have different sensitivity to OA. Within-species and between-population variation was reported by Parker et al. (2011) when they investigated differences between a wild population and selectively bred populations in sensitivity to OA exposure. Their results indicated that sensitivity to rearing conditions may have been different between the populations, as growth rate in the ambient group was higher for the selected group. This may have overestimated the differences in sensitivity to OA exposure alone.

Many factors may contribute to a synergistic response of a species to OA exposure (Table 2). Some have been investigated for early life stages of bivalves: food availability (Thomsen et al., 2010, 2013; Melzner et al., 2011), temperature (Brennand et al., 2010; Courtney et al., 2013; Parker et al., 2010; Talmage and Gobler, 2011), salinity (Dickinson et al., 2012), parental exposure to OA (Parker et al., 2012), fertilization under OA conditions (Parker et al., 2010), and genetic selection (populations or families, Parker et al., 2011, 2012). Additionally, there are other external factors that have not yet been investigated that may impact OA effects through reducing or improving the energy level: rearing volume, diet, day length, UV radiation, etc.

A large part of the work undertaken to investigate effects of OA on marine mollusc larvae has been carried out in volumes of 50 mL–200 L (Table 2). Wernberg et al. (2012) points out that 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 a sufficiently stable rearing environment, and result in negative environmental stress. The volume of exposure tanks in the present study was 38 L, and we cannot rule out that these tanks are too small to keep variations in the rearing environment at a low level. However, the low variation in survival in the control group indicates that the experimental conditions were within acceptable limits.

None of the studies conducted on bivalve larvae are directly comparable with the present study (Table 2). When pCO_2 was relatively similar, the experiments were conducted in stagnant systems with different rearing volumes and densities, or the larvae were fed. As discussed above, all these factors may be synergistic. Larvae in our experiment were reared from eggs of one individual and sperm from three different individuals (i.e. three half-sibling groups) after being well mixed and distributed in equal densities in the experimental tanks. We assume that the half-sibling groups were distributed more or less equally in the tanks, and the variation we found between tanks is most likely not a result of family differences (intra-species differences).

4.4 Larvae survival

The survival of day-7 veligers for the ambient group replicates in this study was 25–46 %. In comparison, the average survival of day-3 veligers (referred to as hatching) in a Norwegian hatchery was 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. In the treatment groups, median survival was reduced by 41–74 %, which was much higher compared to larvae of hard clam (*M. mercenaria*), bay scallop (*A. irradians*), and eastern oyster (*C. virginica*) (Talmage and Gobler, 2009). The highest reduction in survival on day 6 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 were 36 Pa (pH 8.02–8.08). A study on abalone larvae (*Haliotis kamtschatkana*) showed that both survival and growth were reduced by 40 % on day 8, when CO_2 was elevated from an ambient 400 ppm up to 800 ppm (Crim et al., 2011). These variations in survival may indicate inter-species differences, but we cannot rule out that the variation at least partly may be a result of the differences in experimental design (culture volume, water regime, feeding regime).

In an OA study on the Pacific oyster, *C. gigas*, Gazeau et al. (2011) manipulated the A_T levels in one of their treatment groups and showed that the saturation level of CO_3^{2-} had a more severe effect on development and shell growth in oyster embryos than did the pH or pCO_2 level. However, none of the other OA experiments on bivalve larvae have manipulated the A_T level, and thus elevated pCO_2 levels seem to be the most plausible cause of reduced survival in these studies. Although survival of bivalve larvae in OA studies shows a significant variation, a clear trend of a negative effect due to elevated pCO_2 levels or lower pH is found (Table 2). Of six studies calculating survival on larvae, all reported a negative effect on survival (Table 2).

4.5 Larvae shell size

On day 7 larvae size (shell length and shell height) was smaller in the treatment groups compared to the group held in ambient water, but no difference was found between the two mid-treatment groups (807 and 1164 µatm). Reduced shell growth is in accordance with previous stud-

ies (Table 2). Gaylord et al. (2011) found that when CO_2 concentration increased from ambient 380 to 970 ppm, the shell area of mussel larvae (*Mytilus californianus*) on 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 µatm (pH 8.21). Kurihara et al. (2008) also found that the shell length of *Mytilus galloprovincialis* larvae was reduced by about 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}). Parker et al. (2010) found a synergistic effect of elevated pCO_2 and suboptimal temperatures on larvae size of the two oyster species *Saccostrea glomerata* and *C. gigas*, the first being the most sensitive to the exposure.

The reduction in shell growth found in the present study was at the lower end of the scale compared to other studies. This may reflect an inter-species difference, and indicates that *P. maximus* shell growth 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, and keeping the scallop larvae in the same conditions throughout the larval cycle will allow for elucidation of how OA affects growth, survival, and the critical metamorphosis over a longer term.

4.6 Shell deformities

In the present study the percentage 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 supposed to be a result of using gonadal stripping to produce the embryos. In a study on *M. galloprovincialis*, Kurihara et al. (2008) reported abnormal morphology in < 1 % of control larvae, showing that the variation in percentage of normally developed larvae may vary between species, although other conditions may play a significant role.

The percentage of deformed larvae varied highly between the treatments in our study, and the results showed a tendency of more shell deformities with increasing pCO_2 levels. The negative effects observed have been reported also in other OA studies on bivalves (Table 2). Watson et al. (2009) found that in 8-day-old *S. glomerata* larvae, both shell morphology and shell surface characteristics were negatively affected by reduced pH (7.8 and 7.6). Talmage and Gobler (2010) found that the level of CO_2 strongly affected the development of the hinge structure in larvae of *M. mercenaria* and *A. irradians* using CO_2 levels of 250 (pre-industrial), 390 (ambient), 750, and 1500 ppm. They found a decline in size, integrity, and connectedness of the hinge with increasing CO_2 levels. As the authors pointed out, the hinge facilitates opening and closing of the shell, which again is crucial for both feeding and excretion. Losing the ability to perform these basic functions most certainly has a negative effect on larval survival. In the present study, the higher percentage of normally developed hinges on day 7 compared to day 2 in all larvae groups was most likely a result of increased mortality of abnormal larvae from day 2 to 7. The final sample at the end of the experiment did not show a high amount of deformed empty shells to confirm this assumption. However, empty larvae shells are extremely fragile and dissolve fast (and even faster in low-pH water), which may explain why we did not find high numbers of them in the final samples. In future experiments, more frequent sampling may reveal whether the deformed larvae die off throughout the experimental period.

It is known that other factors like self-fertilization, high egg : sperm ratio, temperature, and pollutants may cause an increase in larval deformities (Conroy et al., 1996; Spangenberg and Cherr, 1996; His et al., 1997; Krassoi et al., 1997, Concha et al., 2011; Kobayashi and Kijima, 2010). It has also been reported that factors in the rearing environment such as small rearing volumes, stagnant systems, addition of antibiotics, or pulse feeding may cause larvae deformities in control groups (Kurihara et al., 2007, 2008; Talmage and Gobler, 2009, 2010, 2011; Waldbusser et al., 2010; Gaylord et al., 2011; Gazeau et al., 2011). Although we cannot rule out the effect of small rearing volumes, other factors should play a minor role in the present study as we cross-fertilized the eggs to minimize the amount of self-fertilization, egg : sperm ratio was within the recommended numbers (Martinez et al., 2007; S. Andersen, personal communication, 2012), temperature was within the optimum range (Davenport et al., 1975), we used a flow-to-waste system, and no antibiotics or feed were added.

The ambient water used for the control group in the present study had a pH of 7.98, which was 0.19 units lower than the pH values Talmage and Gobler (2010) used when mimicking the pre-industrial level to investigate past, present and future ocean CO_2 concentrations. They found improved results at pre-industrial pH levels compared to ambient pH levels of 8.05 and 8.04. The shell deformities we found in our control group may therefore at least partly be a result of an already elevated CO_2 level; thus future studies on *P. maximus* should include experimental groups kept in pre-industrial CO_2 conditions to elucidate whether the ongoing OA already may have an impact on scallop larvae.

For the seven OA studies looking at the normal development of bivalve embryos and larvae (a total of seven different species), all reported a negative effect of elevated CO_2 levels (Table 2). Although the results in our study showed a high variation in shell edge deformities between treatment groups, the percentage of hinge deformities increased with increasing pCO_2 levels. As the determination of shell edge deformities can be a bit challenging due to damage caused

by handling the samples, calculating the amount of hinge deformity is probably a better estimate of abnormalities in our study.

4.7 Calcification

In the present study we confirmed that calcein bonded to the calcium in 1–2-day-old trochophore larvae, confirming similar timing for calcification in *P. maximus* larvae as in larvae of *M. mercenaria* and *C. gigas* (Weiss et al., 2002), but at an earlier stage than was suggested by Cass et al. (1998) and Cragg (2006). We were unable to determine the exact time of initiation of calcification in the larvae since the staining period was 18–20 h. 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 exact onset of the calcification process. Comeau et al. (2009) used calcein staining of the pteropod *Limacina helicina* to compare shell extension in animals held at pCO_2 levels of 350 and 760 µatm over a period of five days. Although too few organisms were used to get statistically robust quantitative estimations, the results showed that shell extension in pteropods held at the highest pCO_2 levels were lower compared to those held at 350 µatm. Future OA experiments on *P. maximus* larvae should elucidate whether calcein staining can be used to calculate calcification either as shell extension or size of shell area in larvae held at different pCO_2 levels.

4.8 Concluding remarks and future work

The rather severe effects of elevated future pCO_2 levels on a number of bivalve larvae seem undisputable (Table 2). Talmage and Gobler (2010) showed that the CO_2 levels of today may already affect bivalve larvae negatively compared to pre-industrial levels, and hence, negative effects of OA are happening today rather than in the future. Also, OA should be seen in combination with the increase in global ocean temperature (Blunden and Arndt, 2013). Effects of a temperature increase may amplify the OA effects or reduce them. Talmage and Gobler (2011) showed that the negative effects of elevated CO_2 and temperature on larvae were additive.

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, and also the potential of an evolutionary response in larval development rate. Such studies should involve assessment of the energy budget, take place in facilities that allow a normal development, and include studies on temperature effects. Effects on important processes for survival, such as immune defence functions, feeding rate, and shell mass and strength should also be investigated. More studies using calcein at the earliest larvae stages (day 0–2) are needed to increase knowledge about the onset of the larval shell calcification process, which is crucial to the existence of calcifying organisms.

Supplementary material related to this article is available online at http://www.biogeosciences.net/10/6161/2013/bg-10-6161-2013-supplement.pdf.

Acknowledgements. We thank Ørjan Karlsen for helpful assistance with statistics, and the staff at the Institute of Marine Research – Austevoll Research Station for rearing broodstock, producing algal feed, mixing chemical solutions, and assisting in photographic work. This study was supported by the Institute of Marine Research through project number 13192-04, Ocean Acidification – Scallops.

Edited by: D. Schmidt

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