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Deformities in larvae and juvenile European lobster (*Homarus gammarus*) exposed to lower pH at two different temperatures

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Abstract. The ongoing warming and acidification of the world's oceans are expected to influence the marine ecosystems, including benthic marine resources. Ocean acidification may especially have an impact on calcifying organisms, and the European lobster (*Homarus gammarus*) is among those species at risk. A project was initiated in 2011 aiming to investigate long-term effects of ocean acidification on the early life-cycle of lobster under two temperatures. Larvae were exposed to pCO_2 levels of ambient water (water intake at 90 m depth), medium 750 (pH = 7.79) and high 1200 µatm pCO_2 (pH = 7.62) at temperatures 10 and 18 °C. The water parameters in ambient water did not stay stable and were very low towards the end of the experiment in the larval phase at 10 °C,with pH between 7.83 and 7.90. At 18°, pH in ambient treatment was even lower, between 7.76 and 7.83, i.e. close to medium pCO_2 treatment. Long-term exposure lasted 5 months. At 18 °C the development from stage 1 to 4 lasted 14 to 16 days, as predicted under optimal water conditions. Growth was very slow at 10 °C and resulted in three larvae reaching stage 4 in high pCO_2 treatment only.

There were no clear effects of pCO_2 treatment, on either carapace length or dry weight. However, deformities were observed in both larvae and juveniles. The proportion of larvae with deformities increased with increasing pCO_2 exposure, independent of temperature. In the medium treatment about 23 % were deformed, and in the high treatment about 43 % were deformed. None of the larvae exposed to water of pH > 7.9 developed deformities. Curled carapace was the most common deformity found in larvae raised in medium pCO_2 treatment, irrespective of temperature, but damages in the tail fan occurred in addition to a bent rostrum. Curled carapace was the only deformity found in high pCO_2 treatment at both temperatures. Occurrence of deformities after five months of exposure was 33 and 44 % in juveniles raised in ambient and low pCO_2 levels, respectively, and 21 % in juveniles exposed to high pCO_2. Deformed claws were most often found in ambient and medium treatment (56 %), followed by stiff/twisted walking legs (39 %) and puffy carapace (39 %). In comparison, at high pCO_2 levels 71 % of the deformed juveniles had developed a puffy carapace. Overall, about half of the deformed juveniles from the ambient and medium pCO_2 treatment displayed two or three different abnormalities; 70 % had multiple deformities in the high pCO_2 treatment. Some of the deformities in the juveniles may affect respiration (carapace), the ability to find food, or sexual partners (walking legs, claw and antenna), and ability to swim (tail-fan damages).

1 Introduction

The world's atmosphere is increasingly becoming more saturated with the concentration of CO_2 as carbon emissions from burning fossil fuels keep increasing (IPCC, 2007; Caldeira et al., 2005). Atmospheric CO_2 is currently around 380 ppmv, but is predicted to increase to 780 ppmv and 1200 ppmv by 2100 and 2200, respectively (IPCC, 2007). Increased absorption of atmospheric CO_2 into the marine environment leads to an increase in total dissolved inorganic carbon (DIC), which changes the chemistry and acid-base balance and results in a decreased seawater pH (Dickson et al. 2007). Currently the global average seawater pH is about 8.05 units, and is associated with a DIC of 2026 (Fabry et al., 2008). It is predicted to drop by 0.3 to 0.4 pH units by 2100 (Orr et al., 2005; Feely et al., 2009). At the same time, depending on the emission scenarios, ocean temperature in

the upper 100 m is predicted to increase by 0.6° (RCP2.6) to 2 °C (RCP8.5) by 2100, (Blunden and Arndt, 2013). The absorption of CO_2 by the ocean and ocean acidification (OA) occurs mostly in the upper 100 m, and varies with latitude and temperature (Orr et al., 2005; Fabry et al., 2008).

Regional monitoring of DIC at Station M in the eastern Norwegian Sea shows a value of 2140 (Skjelvan et al., 2008), which is above the global average of 2026. This agrees with recent modeling that shows CO_2 uptake is higher and pH lower in the eastern Norwegian Sea (Olsen et al., 2006). pH levels will decrease with water depth at which aragonite becomes undersaturated, from ∼ 100 to 50 m by 2100 at high latitudes (Fabry et al., 2008). Marine organisms in colder regions are therefore at a greater risk of being affected by the effects of both warming and OA, especially calcifying marine organisms. One such species is an important predator, the European lobster *Homarus gammarus*.

H. gammarus is found along the continental shelf in the northeast Atlantic, which extends from the warm waters off Morocco to the colder areas near the Arctic Circle, i.e. Tysfjord and Nordfolda (68° N) (Agnalt et al., 2009). This distribution covers a large latitudinal and temperature range. With CO_2 absorption varying greatly with latitude and temperature, the effects of acidification may vary greatly for subpopulations across the distribution range. The life cycle of *H. gammarus* consists of four larval stages, a juvenile stage, a sub-adult stage (∼ 50 mm carapace length: CL) and adult of > 60 mm CL (Factor, 1995); larvae are pelagic in the first three stages (stage 1–3), after which they settle during larval stage 4. Little is known about the benthic stages of *H. gammarus* juveniles less than 40 mm CL in the wild (Linnane et al., 2001). The European lobster is esteemed as a valuable marine resource and has supported the coastal fishery in northern Europe for several centuries (Agnalt, 2008). In the 1960s, lobster populations in Norway were depleted below sustainable levels and due to low recruitment, the recovery has been slow. Any additional factors that reduce recruitment and population size further may push this species to the brink of extinction in these areas.

Only one study has investigated the effects of OA (pCO_2) in *H. gammarus*, focusing on larvae at the predicted future scenario of pCO_2 1200 µatm (Arnold et al., 2009). Calcification was significantly reduced in stages 3 and 4, with no direct effect on growth observed. The decrease in calcification observed in stage 4 at a pCO_2 of 1200 µatm may have been due to energy being channelled towards growth and possibly acid-base regulation (Arnold et al., 2009). Growth in crustaceans requires replacement of the old exoskeleton by a new and larger exoskeleton, in a process called moulting, occurring more frequently in juveniles compared with adults. Moulting is highly temperature dependent, making juveniles vulnerable to temperature changes (Waddy et al., 1995). Moulting also involves depositing $CaCO_3$ to harden the shell, which is energetically costly and therefore puts great physiological stress on the animal. Low pH resulting from OA increases physiological stress and may be devastating to moulting juveniles already under metabolic stress. Warmer temperatures, predicted to co-occur with increased OA, may have its added metabolic stress on juvenile lobsters (as seen in the crab *Hyas araneus*) if thermal tolerance limits are exceeded (Walther et al., 2010). Ries et al. (2009) investigated the impact of OA on a range of benthic marine calcifiers and found that *Homarus americanus* juveniles had the highest net calcification at high pCO_2 levels (2856 ppm). The increase in calcification is thought to be initiated by actively increasing pH at the calcifying centres, therefore reducing H^+ and converting bicarbonate (HCO_3^-) to carbonate (CO_3^{-2}). CO_2-induced acidosis in crustaceans is usually compensated for by increasing bicarbonate production (Truchot, 1978; Pörtner et al., 2004; Spicer et al., 2007). Bicarbonate production is energetically costly and may be reduced over the long term if energy reserves are low.

The one study on OA in *H. gammarus* and studies concerning other crustacean species suggest different impacts occurring in different stages of the life cycle. The synergistic effects of warming and OA on the life cycle of *H. gammarus* (and other lobster species) are unknown and urgently need to be studied. A project was therefore initiated in 2011 aiming to investigate long-term synergistic effects of temperature and projected increases in ocean acidification on the early life cycle of lobster, i.e. the larval and juvenile phase of *H. gammarus*. Here we use 18 °C, similar temperatures to Arnold et al. (2009), which are optimal for hatching and growth (Wickins and Lee, 2002; Kristiansen et al., 2004) and 10 °C which are less optimal conditions (Schmalenbach and Buchholz, 2013). The temperatures chosen are not a likely climate change scenario but a physiological test.

2 Material and methods

Experiments combining OA with temperature were conducted at IMR-Matre (60°52′ N, 05°35′ E) over a period of five months, lasting from 28 September 2011 to 22 March 2012. Raw water was pumped from 90 m depth, thus representing ambient water. Each experimental unit consisted of a 400 L tank of 0.87 × 0.87 × 0.53 m. Six of these units were used for the ambient, medium (750 µatm) and high (1200 µatm) pCO_2 treatment run at two temperatures (10°C and 18°C). For the larval rearing, two 40 L incubators (Hughes kreisel) were placed in each unit (12 in total, Fig. 1a). For on-growing, the juveniles were kept individually in trays, placed in the experimental units (Fig. 1b). From 5 months of age, the surviving juveniles were continuously monitored but under ambient conditions at 14 °C, until 25 October 2012, by which time they were about 1 year old. The larvae and juveniles were all kept in 8 to 10 h light and 16 to 18 h dark.

2.1 Water parameters

The salinity during the course of the experiment was on average 33.7 ± 0.2 ppt. Temperature was run at 10 and 18 °C, to simulate a lower limit and an optimum threshold for homarid lobsters, respectively (Wickins and Lee, 2002; Kristiansen et al., 2004; Schmalenbach and Buchhoz, 2013). Initially a middle temperature of 14 °C was also included, but due to the limited number of larvae available this had to be excluded. The experiments were run in ambient water, believed to be at a current pCO_2 of 380 µatm occurring in the natural oceanic environment; treatments with a medium pCO_2 of approximately 750 µatm and with a high pCO_2 aiming at 1200 µatm, to simulate low and medium OA scenarios predicted for 2100 and 2200, respectively. Multiple 2×2 AGA 25 kg gas bottles were used to induce CO_2 into two cones, creating seawater with pH = 6. From each of these cones, CO_2-rich water was mixed with oxygen-rich seawater and the flow rates from the cones were regulated by controllers to obtain the desired seawater quality; pH = 7.79 in medium pCO_2 treatment and pH = 7.62 in high pCO_2 treatment. Water from the enclosures were supplied to and circulated through the experimental tanks. Temperature, pH and oxygen were monitored continuously in each experimental tank by probes connected to a computer. The pH was measured several times daily using Orbisint CPS11D from Endress + Hausser electrodes, using the National Bureau of Standards (NBS) scale. pH was calibrated once a week with buffers of pH = 4.0 and 7.0. In addition, calibration was also made by using a spectrophotometry Hitachi U-2900 connected to a Refrigerated Heating Circulator Julabo F12 combined with temperature sensors TD301A from SAIV A/S. These measurements deviated from 0.001 to 0.053 from pH_{NBS} and were considered acceptable for this study. Samples for analysis of CO_2 content in seawater in µatm, total alkalinity (A_t) and total dissolved inorganic carbon (C_t) were collected in 350 ml amber glass bottles with minimal headspace. 300 µL of saturated $HgCl_2$ solution was added to preserve the sample. Aragonite and calcite saturation state (Ω) and pH on the total hydrogen ion scale (pH_t) were then calculated.

In the medium pCO_2 treatment the content of pCO_2 was 727 ± 12 µatm, and correspondingly 1198 ± 157 µatm in the high pCO_2 treatment (Table 1), combined for both temperatures. The water quality in ambient water, i.e. intake of raw water at 90 m depth, did not stay stable. At 10 °C, pH in the larval experiments was stable at an average 8.01 from 28 September until 11 November, then dropped to 7.92 and even dropped further and was between 7.83 and 7.90 towards the end of the experiment (Fig. 2). In ambient water at 18 °C the pH was relatively stable but at 7.80 ± 0.02 throughout the larval phase. In other words, pH was above 7.9 only in two-thirds of the larval phase at 10 °C. pH was relatively stable in the experiments run at medium and high pCO_2 at both temperatures, however with a few outliers (Fig. 2). In the juvenile experiments pCO_2 in ambient water was higher than expected (Fig. 3) and only slightly lower than the medium pCO_2 treatment. In the experiments monitoring the surviving juveniles from the exposed treatments grown in ambient water until 8 months of age, pH varied around 7.95 to 7.96 (data not shown).

Table 1. Seawater parameters during the OA experiment. A_t: total alkalinity; C_t: total dissolved inorganic carbon. Analysis of nutrition gave estimates of nitrate (12.3 µmol kg^{-1}), phosphate (1.3 µmol kg^{-1}) and silicate (7.1 µmol kg^{-1}) used in the calculations.

	Ambient	Medium pCO_2 treatment	High pCO_2 treatment
pCO_2 (µatm)	692 ± 26	727 ± 12	1198 ± 157
Salinity (ppt)	33.37 ± 0.12	33.37 ± 0.12	33.34 ± 0.09
A_t (µmol kg^{-1})	2314.4 ± 2.5	2310.3 ± 1.1	2318 ± 14.3
C_t (µmol kg^{-1})	2168.3 ± 6.6	2171.3 ± 1.5	2266.4 ± 31.8
HCO_3^-	2028.5 ± 9.2	2034.6 ± 2.6	2154.7 ± 35.4
CO_3^{-2}	116.2 ± 3.7	112.0 ± 1.5	66.1 ± 11.0
pH	7.84 ± 0.01	7.82 ± 0.01	7.62 ± 0.05
$\Omega_{calcite}$	2.81 ± 0.09	2.71 ± 0.04	1.60 ± 0.27
$\Omega_{aragonite}$	1.81 ± 0.06	1.75 ± 0.02	1.02 ± 0.18

2.2 Brood stock

Ovigerous females were collected from the *H. gammarus* population in Øygarden (60°35′ N; 4°50′ E) during the commercial fishing season October to November 2011, and transported to IMR-Matre. They were acclimatized at 6 °C in individual 75 L tanks ($52 \times 52 \times 28$ cm) in a CT room with the lighting set to a 12 h dark:12 h light cycle. Lobsters were fed frozen shrimps and fish twice a week. The CO_2-control system was operative in late Septmeber 2011, and the hatching was postponed accordingly. In early September the temperature was slowly increased to 18 °C, to induce hatching that commenced 28 September 2011. Of a total of 14 females, only four had eggs that hatched during the experimental period. The sizes of the ovigerous females were 91, 110, 113 and 135 mm carapace length (CL; measured as the distance from the posterior rim of the eye socket to the posterior edge of the carapace). The females hatched 15 160 larvae in total.

2.3 Larval rearing and sampling

Each of the individual tanks with the ovigerous females had an overflow through a 20 mm water hose leading to separate containers equipped with a filter to retain the hatched larvae. Larvae normally hatch during late night/dawn and were collected from the outflow containers each morning, counted and transferred in equal numbers to the 40 L upstream incubators (plankton Kreisler; Hughes et al., 1974). However, all females were not hatching at the same time, thus we chose to run the experiments with pCO_2 treatments at 10 °C before commencing at 18 °C. The incubators were supplied with

Fig. 1. Overview of the experimental setup during the **(a)** larval (two incubators as parallels in each tank unit) and in the **(b)** juvenile phase with trays with individual compartments (several trays in each tank unit).

11 L seawater per minute. The larvae were fed daily with frozen *Artemia* sp. Larvae hatching over a period of 3 days were mixed in the same incubator. Larvae with larger difference in age were not mixed due to increased risk of cannibalism. Maximum density for each incubator was set to 1000 larvae, or 25 larvae per litre. Each treatment, i.e. temperature and CO_2 had two replicates (as shown in Fig. 1). Every third day the incubators were treated with Chloramid-T to control growth of the bacterium *Leucothrix mucor*, as previously experienced in other lobster-rearing systems (A.-L. Agnalt, personal communication, 2011.). The larvae were staged 1 to 4, according to Sars (1875) and Herrick (1909). Care was taken to look for intermediate larval stages, as this has been observed in American lobster (*H. americanus*), especially between stage 2 and 3 and between stage 3 and 4 (Templeman, 1936; Wells and Spraque, 1976; Charmantier and Aiken, 1987). Intermediate larval stages have also been observed in hybrids, i.e. offspring from female American lobster and male European lobster (Agnalt, unpublished data).

A total of 10 larvae at each development stage (1 to 4) from each incubator were sampled for measurement of CL and dry weight. At each stage, larvae had been exposed for a minimum of three days before sampling. All measurements of CL were recorded using a dissecting microscope. Dry weights of individual larvae were recorded after 3 days of drying in Termaks dry oven at 60 °C, and recorded to the closest microgram (mg) using a Mettler Toledo scale (AG204 Delta Range). As many of the samples as possible were processed while the larvae were still alive. When the time schedule was too tight the larvae were frozen individually to be processed at a later stage. To utilize the system fully, whenever an incubator was terminated, and provided there were still newly hatched larvae available, another production line was started. Two batches of larvae production was made, with consecutive sampling, in incubator 2a, 2b, 3a, 14b, 15a and 15b (Fig. 1). Unfortunately, during storage the freezer containing many of the frozen samples of the larval stages broke. For these samples, CL and dry weight were recorded for as many larvae as possible. For the experiments run at 10 °C, 185 out of 216 sampled larvae have size recordings, and correspondingly at 18 °C, 224 out of 323 sampled larvae.

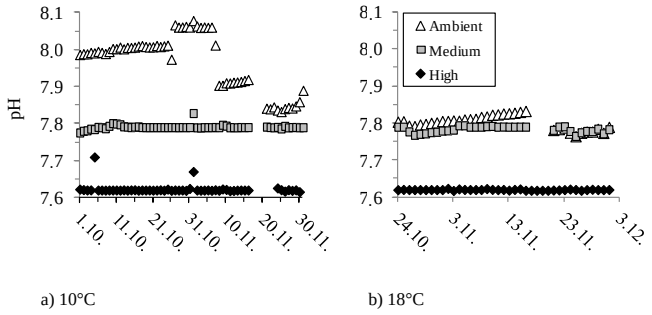

Fig. 2. Variation in pH in the larval phase run in ambient, medium pCO_2 and high pCO_2 at **(a)** 10 °C and **(b)** 18 °C in 2011. Ambient is raw water from 90 m depth. Note that the 10 °C experiment started before 18 °C.

2.4 Long-term exposure of juveniles at five months

While still pelagic, stage 4 larvae were collected one by one and transferred to trays consisting of single-cell compartments, made of black PVC plastic. Each tray consisted of 30 to 40 individual compartments with perforated bottoms (1 mm × 1 mm holes) to ensure water flow. Three to four trays were placed together in 400 L units (87 × 87 × 53 cm). Each unit was given water quality according to ambient, medium pCO_2 or high pCO_2 treatment. Water flow was set to 18 L per minute. The lobster juveniles were fed commercially produced pellets (2 mm), patented by Norwegian Lobster Farm (http://www.norwegian-lobster-farm.com/no) and produced by Nofima (http://www.nofima.no/). Whenever a juvenile moulted, the old exoskeleton was not removed from the compartment. On 22 March, CL were recorded for each of the surviving juveniles.

2.5 Continued monitoring of surviving juveniles in ambient water until 1 year old

From five months the juveniles were all kept in ambient water, i.e. the water quality in the raw water intake, at 14 °C from 22 March to 25 October 2012. The purpose was to verify if the deformities observed were retained or lost through moulting when kept in water of higher pH (between 7.95 and 7.96). The lobster juveniles were fed commercially produced pellets (5 mm), patented by Norwegian Lobster Farm (http://www.norwegian-lobster-farm.com/no) and produced by Nofima (http://www.nofima.no/). At the end of the experiment, CL was recorded for each surviving juvenile.

2.6 Statistics

To determine whether parallels could be pooled for each pCO_2 treatment and each temperature we used Mann–Whitney U test. A Kruskal–Wallis test was used to determine if there were significant differences between lobsters undergoing the different pCO_2 and temperature treatments.

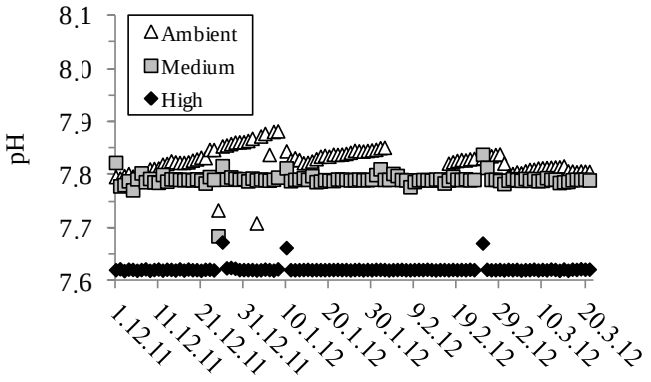

Fig. 3. Variation in pH in the juvenile phase in ambient, medium pCO_2 and high pCO_2 until 22 march 2012 in the experiments run at 18 °C. Ambient is raw water from 90 m depth. Note that only juveniles in 18 °C were monitored, as non larvae reached a viable stage 4 in 10 °C.

One-way ANOVA was used to analyse length frequency of the juveniles raised in the different pCO_2 treatments.

3 Results

3.1 Growth

Growth was very slow at 10 °C, irrespective of pCO_2 level, and after 5 weeks none of the larvae had moulted into stage 4. No larvae reached stage 3 in the medium pCO_2 treatment. Eventually three larvae reached stage 4, in high pCO_2 treatment only, but died within the following two days. At 18 °C, development from stage 1 to 4 lasted from 14 to 16 days independent of pCO_2 treatment as predicted under optimal conditions. Of the 409 larvae investigated, none were found in intermediate stages.

At 10 °C, carapace length at stage 1 and stage 3 did not differ significantly between pCO_2 treatments ($p > 0.5$, Fig. 4.). However, there were significant differences at stage 2 ($p < 0.05$), and the larvae produced in medium pCO_2 treatment were slightly smaller in CL compared with ambient and high pCO_2 treatment. At 18 °C, CL was significantly different at stage 2 and stage 3 ($p < 0.05$, Fig. 4.). At stage 2, larvae reared in medium pCO_2 treatment were on average larger than the larvae reared in the other treatments, but at stage 3 they were smaller. Dry weight differed significantly between pCO_2 treatments at stage 1 and 2 at 10 °C, and stage 2 and 3 at 18 °C ($p < 0.05$, Fig. 4). Larvae raised in ambient water at 10 °C were lighter in weight at stage 1 and heavier at stage 2 compared with the treated larvae. At 18 °C, the stage 2 larvae raised in ambient water were slightly heavier than the other treatments but at stage 3 the high pCO_2 treatment larvae were heavier. In other words, there was no consistence and clear effect of pCO_2 treatment on either CL or dry weight.

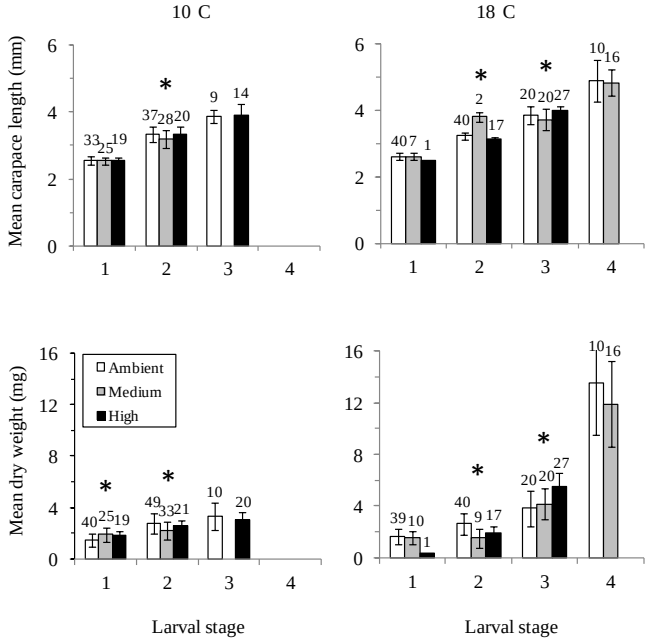

Fig. 4. Mean carapace length (mm) and mean dry weight (mg) with one standard deviation at each larval stage of *Homarus gammarus* undergoing ambient, medium pCO_2 and high pCO_2 treatment run at 10 and 18 °C. Number of observations in each stage is given above the column. Note that at 10 °C only three larvae reached stage 4 (high pCO_2 treatment), but they only survived for two days. Ambient is raw water from 90 m depth. Star indicates significant differences based on Kruskal–Wallis non-parametric test at $p = 0.05$ level.

As only larvae raised at 18 °C successfully moulted into stage 4, this was the only temperature for which long-term exposure to pCO_2 could be monitored. Survival from stage 4 until 5 months age averaged 46 % in ambient, 17 % in medium and 61 % in high pCO_2 treatment. In total 148 juveniles survived. There were significant differences in CL as a result of pCO_2 treatment ($p<0.05$, Fig. 5), but the sample size was however low at the medium pCO_2 treatment ($N = 16$), accounting for the difference since there were no significant differences between ambient and high pCO_2 treatment ($p > 0.1$, Fig. 5).

3.2 Deformities

Deformities, i.e. a difference in the shape of a body part or organ compared to the normal shape was found in the larvae (Table 2) and the juveniles (Table 3). The morphological abnormalities in the larvae were classified as curled carapace (Fig. 6), damages to the tail fan or that the rostrum was bent (Table 2). No larvae suffered multiple deformities. The most affected part was curled carapace, occurring in 59 % of the deformed larvae when combining all treatments. Bent rostrum was found in 27 % and damages to the tail fan in 14 % of all the deformed larvae. The deformities in the larvae were

Table 2. Classification of deformities found in larval stages 1 to 4 in European lobster (*Homarus gammarus*) exposed to elevated pCO_2 levels.

Category	Organ affected	Description
Curled carapace	Carapace	The carapace was curled at the edge, often forming ridges penetrating the side of the carapace.
Tail-fan damages	Uropod	Damages to parts of the tail fan, or even lacking one or both of the tail fans.
Bent rostrum	Rostrum	The rostrum was bent, as if not yet straighten out after hatching.

observed and described in live samples only. It was difficult to distinguish misshapes as described in Table 2 from artefacts due to the freezing process. Hence frozen samples were not included in the calculations below.

The proportion of larvae with deformities increased with increasing pCO_2 exposure, but was similar across the two temperatures 10 and 18 °C. In the medium treatment, 22 and 24 % were deformed at 10 and 18 °C, respectively. At the high treatment as much as 42 and 45 % were deformed, respectively. An overall 12 % of the larvae in ambient water raised in 18 °C developed deformities, though it is important to note that pH in ambient treatment was only slightly higher than in the medium pCO_2 treatment. At 10 °C, 5 % of the larvae ($N = 2$) were deformed, but these two specimens were actually sampled after 11 November when pH dropped to below 7.9. None of the larvae exposed to pH above 7.9 developed deformities. Curled carapace was the most common deformity (45 %) found in larvae raised in medium pCO_2 treatment at 10 °C, followed by damages in the tail fan (33 %) and bent rostrum (22 %). Concurrently, in the high pCO_2 treatment curled carapace was the only deformity. In the deformed larvae raised at 18 °C and in ambient treatment, curled carapace (33.3 %), damages to the tail fan (33.3 %) and bent rostrum (33.3 %) were equally distributed. Concurrently, in the medium treatment half of the deformed larvae had a bent rostrum, 38 % a curled carapace and 12 % were found with damages in the tail fan. Curled carapace was the only deformity found in the larvae undergoing high pCO_2 treatment.

Of the 148 juveniles that survived after five months, 41 were classified as morphologically deformed (see Table 2). Overall, 33 and 44 % of the juveniles in ambient and medium pCO_2 treatments, respectively, were deformed compared with 21 % in juveniles exposed to high pCO_2 (Fig. 8). In ambient and medium pCO_2 treatment, deformed claws were most often found (56 %), followed by stiff/twisted walking legs (39 %) and puffy carapace (39 %). When the juveniles

Table 3. Classification of deformities found in juvenile stages of European lobster (*Homarus gammarus)* exposed to elevated pCO_2 levels.

Category	Organ affected	Description
Puffy carapace	Carapace	Carapace puffy/swollen, or up-folded on one side, often leaving some parts of the gills exposed.
Stiff/twisted walking legs	2–5 pereopod	The joints were fused together as if the joints were over-calcified. The entire pereopod leg was like one stiff piece, sometimes "frozen" in an arbitrary/twisted position.
Misshaped claw	1st pereopod/cheliped	Various shapes of the cheliped, but most often twisted, deviating from normal.
Bent rostrum	Rostrum	The rostrum was bent, as if not yet erected after hatching.
Tail-fan damages	Uropod	Damages to parts of the tail fan, or even lacking one or both of the tail fans.
Twisted abdomen	Abdomen	Abnormal shape of the abdomen as if some of the segments were once broken and then grown back in the wrong shape.
Stiff antenna	2 antenna	Segments of the antenna were fused, as if the joints were over-calcified. Felt "stiff" when touching. Difficult to observe when animal was out of water.

from ambient and medium pCO_2 treatment had deformed claw(s) about 54 % had also developed stiff/twisted walking legs. In comparison, at high pCO_2, 71 % of the deformed juveniles had developed a puffy carapace. Of these, 24 % had also developed deformed claws. Overall, about half of the deformed juveniles from the ambient and medium pCO_2 treatment had developed two or three different abnormalities (Fig. 10). In comparison, 70 % of the deformed juveniles in the high treatment had multiple deformities.

At one year of age, 76 of the 148 juveniles had survived. Mortality was equally high for those juveniles that had 5 months' exposure in ambient or in high pCO_2. Overall, 28 % of the juveniles were deformed. The most common occurring deformities (28 %) were puffy carapace with stiff/twisted walking leg, as illustrated in Fig. 10. Of the 40 deformed juveniles found 22 March 2012, only 12 had survived another seven months. Of these, six were still deformed (four of the juveniles even developed at least one additional deformity). In other words, 50 % of the survivors had managed to recuperate, most likely through moulting. However, of the 108 juveniles classified as normal at five months of age, 15 had developed deformities seven months later.

4 Discussion

Deformities were observed both in the larval and juvenile phase in European lobster when exposed to higher pCO_2 from hatching. The proportion of larvae with deformities increased with increasing pCO_2 exposure and was similar across the two temperatures 10 and 18 °C. In high exposure as much as 45 % of the larvae developed deformities. After five months of exposure, 44 and 21 % of the juveniles were deformed in medium and high pCO_2 treatment, respectively. Deformities in lobster larvae and juveniles have not previously been reported, either in European or American lobster, although the scientific community have long-term experience in husbandry of these two species (Gruffydd et al., 1975; Capuzzo and Lancaster, 1979; Latrouite and Lorec, 1991; Addison and Bannister, 1994; Uglem et al., 1995; Agnalt et al., 1999, 2004; Nicosia and Lavalli, 1999; Linnane et al., 2000; Jørstad et al., 2001; Wickins and Lee, 2002; Kristiansen et al., 2004; Jørstad et al., 2005; Agnalt, 2008; Arnold et al., 2009, Ries et al., 2009, Schmalenbach et al., 2009, Keppel et al., 2012). Wickins et al. (1995) did report moulting abnormalities in European lobster larvae in relation to testing different diets, but with no further description

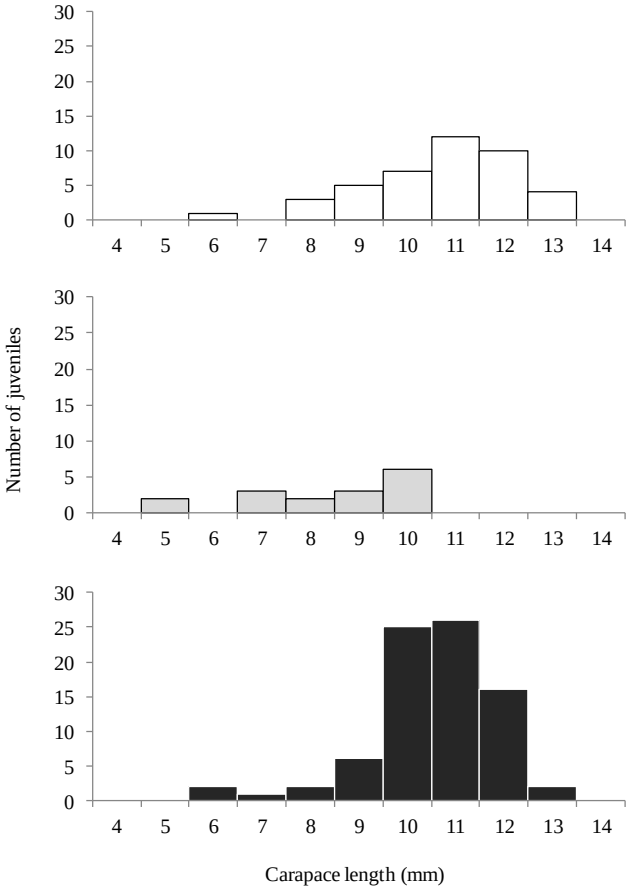

Fig. 5. Carapace length (mm) frequency of juvenile *Homarus gammarus* raised in 18 °C, from newly hatched larvae until 5 months of age in **(a)** ambient water, **(b)** medium pCO_2 treatment and **(c)** high pCO_2 treatment. Size recordings were made 22 March 2012.

Fig. 6. A stage 3 *Homarus gammarus* larvae raised in an environment with pH lower than 7.9. The carapace is curled, leaving "curls" on the side (indicated with the arrow). This is classified as a deformity.

of what the abnormalities were. In aquaculture, hatchery-induced changes due to feed, tank design and/or substrate have been described for a number of fish and shellfish species (Olla et al., 1998; Svåsand et al., 1998; Tsukamoto et al., 1999; and references therein). In shellfish, most changes documented are morphological, e.g. lower shell strength in the great scallop (*Pecten maximus*) (Grefsrud and Strand, 2006) and queen conch (*Strombus gigas*) (Stoner and Davis, 1994), lack of spines in top shell (*Trochus niloticus*) (Purcell 2002) or lack of differentiation in the claws in lobster Homarus spp. (Govind and Pearce, 1986). Deformities of 40 to 58 % have also been found in wild populations of shrimps of the genus *Palaemon* in the Gironde estuary in France (Béguer et al., 2008, 2010). The deformities (wrinkled or bent carapace, bent rostrum and damages in the tail fan) were reported to affect adult individual mortality and egg production. The cause of these deformities was not identified, although stress, pollution or even elevated pCO_2 levels may be explanatory factors. In future studies it will be vital to differentiate deformities caused by ocean acidification from effects due to hatchery production or other environmental factors.

Why are we so sure that the deformities observed in this study were due to high exposure pCO_2? The Institute of Marine Research has since the early 1990s hatched and produced larvae and juvenile European lobster for various studies such as, e.g. stock enhancement (Agnalt et al., 1999, 2004; Agnalt 2008), fitness and genetic studies (Jørstad et al., 2001; Jørstad et al., 2005), carrying capacity (Agnalt, unpublished data) and conditioning juveniles for release purposes (Agnalt, 2013; Aspaas, 2012; Trengereid, 2012). We have only in a few occasions found that the first pair of peripods (claws) was misshaped but this trait was lost after one or two moults. None of these misshapes looked like the claw deformities found in the present study. Assessing deformities demands experience with observing larvae and juveniles, as subtle trait changes might be missed. In the present study, deformities in the larval phase were found in the carapace (termed "curled" in larvae and "puffy" in juveniles), tail fan or in the rostrum (termed "bent rostrum") (Tables 2 and 3). Inexperienced personnel might miss these traits. In this study, measurements were made by one person to ensure consistency and the individuals were classified blindly. Experience should be accounted for also in future studies, whenever possible.

Our study shows that deformities occur in lobster larvae raised in pCO_2 levels higher than 727 µatm, independently of temperatures being 10 or 18°C. Deformities due to OA has been documented on the embryonic in some marine invertebrate species, often resulting in low or even no hatching success (Parker et al., 2009; Kawaguchi et al., 2010; Byrne, 2011), but also in the larval stages in a few species (Kurihara, 2008; Byrne et al., 2011, and references therein). Whiteley (2011) stated that the exoskeleton of planktonic decapod larvae is unmineralised and elevated pCO_2 should therefore

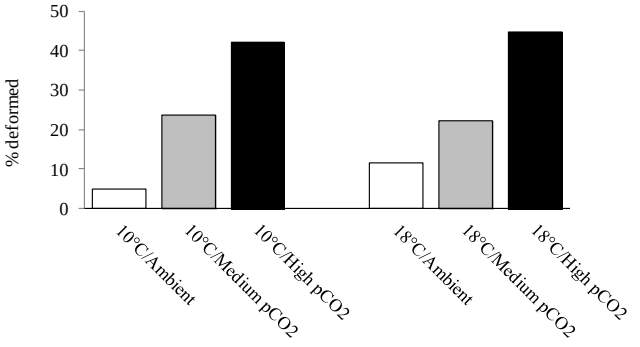

Fig. 7. Percentage of *Homarus gammarus* with deformities in the larval phase in the different temperature, 10 and 18 °C, and pCO_2 treatments (ambient, medium and high).

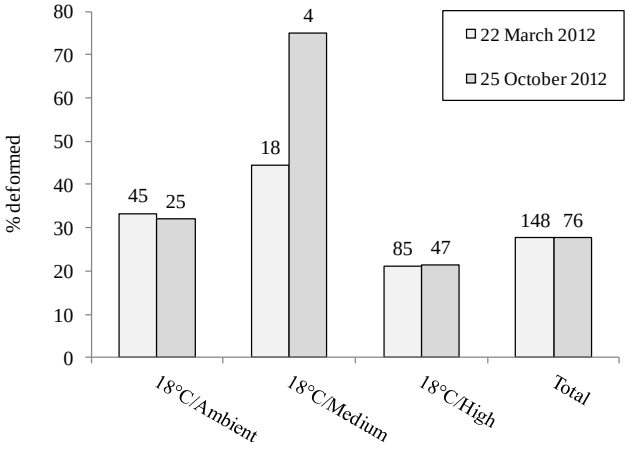

Fig. 8. The percentage of juveniles with deformities. Light grey bar represents juveniles surviving five months of pCO_2 exposure (ambient, medium and high) and darker grey bar the juveniles after another seven months in ambient water only. Total number of juveniles in each category is given above each column.

not affect larval conditions. However, Arnold et al. (2009) analysed the calcium and magnesium concentrations per surface area of the carapace of *H. gammarus* larvae. They found significant reductions at elevated pCO_2 of 1200 µatm in stage 4 larvae; calcium decreased from 0.24 to 0.13 µg mm^{-2} and magnesium decreased from 0.019 to 0.012 µg mm^{-2}. Calcium and magnesium seem to be important mineral components in the exoskeleton of the lobster larvae. However, Mg-$CaCO_3$ is more soluble than pure calcite or aragonite (Andersson et al., 2008) and since $\Omega_{Aragonite}$ was at the lowest 1.02 in this study, elevated pCO_2 could definitely have an effect on e.g. formation of the new exoskeleton. A thinning of the shell has been reported in blue mussels *Mytilus edulis* and Pacific Oyster *Crassostrea gigas* (Gazeau et al., 2007; Melzner et al.; 2011). A thinner shell in lobster larvae may not be strong enough to keep its shape, especially when covering organs like the gills where water flow continuously and applies pressure on the shell. This may explain the "puffy" carapace observed in the present study.

In the high pCO_2 treatment carapace was the most affected organ, both in larvae and in the juveniles. In the juveniles, the deformities generally affected carapace (puffy or swollen, thus often leaving part of the gills exposed), walking legs (stiff), claws (twisted), abdomen (stiff joints), tail fan and even antenna (stiff). The latter is of vital importance for lobster communication, including finding a partner (Johnson and Atema, 2005). Fewer deformities were found in juveniles exposed to high pCO_2 compared with medium exposure. However, those affected had also developed multiple deformities, i.e. more severe damages. We found that damages to the tail fan could not be repaired through moulting, while walking legs and "puffy" carapace would become normal after several moultings. The exoskeleton in sub-adult and adult lobster can be divided into three layers: epi-, exo- and endocuticle (e.g. Sachs et al., 2006; Bosselmann et al., 2007; Romano et al., 2007; Al-Sawalmih et al., 2008; Sachs et al., 2008; Fabritius et al., 2009). The exoskeleton of *Homarus* sp. consists of chitin, protein, calcium, magnesium, phosphate and a few other compounds (Ba, Mn, Sr), although variable in composition in the different layers (Al-Sawalmih et al., 2008; Kunkel et al., 2012). The epicuticle is particularly rich in calcium, magnesium and phosphate. The outermost exocuticle consists of a thin calcite-containing layer while the rest is fully mineralized, mostly with amorphous calcium carbonate that is highly soluble and acts as a transient source of calcium. The endocuticle consists of crystalline calcite. Moulting is a complex process (Greenaway, 1985; Dillaman et al., 2005; Politi et al., 2010), and for instance, reabsorption of calcium occurs from the old exoskeleton before it is shed. The new exoskeleton is uncalcified and rapidly needs to harden by deposition of calcium carbonate. In other words, elevated pCO_2 can affect various cuticle layers and various stages of the moulting cycle. Thus, the deformities may have been caused by irregularities in the depositing of Mg-$CaCO_3$ in the shell. Too high deposits, a possible compensation for low hemolymph pH (acidosis), may have resulted in the stiff walking legs observed in our study. Whereas too little depositing of $CaCO_3$ in the shell may result in the incomplete formation of certain structures in the exoskeleton. This could be related to depleted energy resources (to maintain homeostasis) required for converting HCO_3^- to CO_3^{2+} and therefore the depositing of enough $CaCO_3$.

No consistent and clear effect of pCO_2 treatment was found in this study on neither carapace length, or on dry weight. This is coherent with observations with the same species by Arnold et al. (2009). Neither was moulting frequency affected by increased acidification in this study. However, Keppel et al. (2012) found decreased carapace length with increased pCO_2 in the closely related *H. americanus*, and the moulting cycle was prolonged with about 2 days in

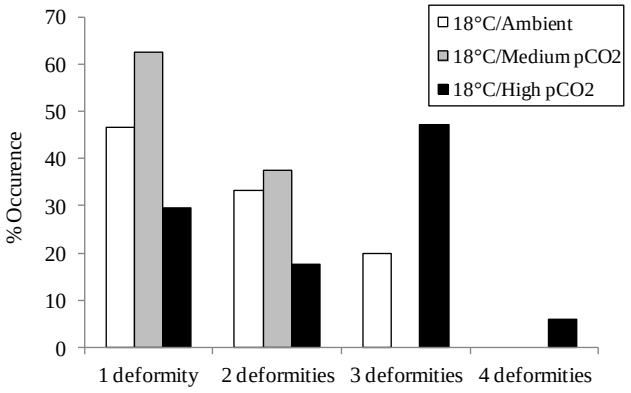

Fig. 9. The percentage of lobster juveniles with single or multiple deformities when exposed to elevated levels of pCO_2 (ambient, medium and high) for five months.

larvae raised at pH = 7.7 compared with pH = 8.1. It seems that the growth and moulting cycle is affected in *H. americanus* (Keppel et al., 2012), while larvae and juveniles of *H. gammarus* maintain growth rates at the cost of mineralization of the exoskeleton.

A major concern with the present study was the very low pH with corresponding high pCO_2 (average 692 ± 26 µatm) found in ambient water taken from a locality in Masfjorden at 90 m depth. Compared to pH in ambient water at IMR's research stations Austevoll (pH 7.98, Andersen et al., 2013) and Parisvatn (pH 8.11; Agnalt, personal communication) located in outer coastal areas in the same region, the water in Masfjorden seems to have quite a large natural variation and may drop to levels well below global average pH of 8.05 (Fabry et al., 2008). European lobster is not normally found at the depths of 90 m at this locality in Masfjorden, but is found in the fjord system at shallower depths. Knowledge about the pH in the Norwegian fjord systems and how it fluctuates between seasons and years is scarce. However, measurements from other locations in the Northern Hemisphere shows that pH in coastal areas can be highly variable and as low as 7.7–7.6. Barton et al. (2012) showed that pH in Oregon coastal water varied from 7.6 to 8.2 in the early summer of 2009. Newton et al. (2012) reported a pH of 7.72 at 100 m depth in Puget Sound and the Strait of Juan de Fuca, on the East Coast of the USA during February 2008. Even in the "closer to home" North Sea, there are already indications that the level of pH is variable and decreasing (Olsen et al., 2006; Blackford and Gilbert, 2007; Bellerby et al., 2005). Based on these studies, we assume that fjord systems also are affected by the ongoing ocean acidification and may explain the low ambient conditions in Masfjorden. However, more effort is needed to verify this hypothesis.

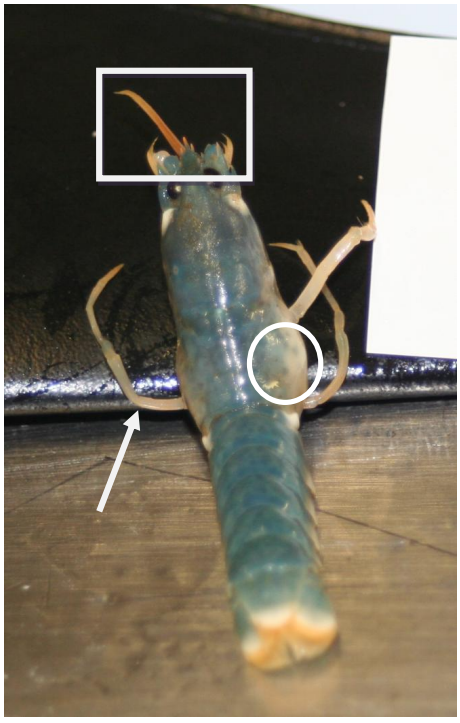

Fig. 10. *Homarus gammarus* juvenile, one year of age, exposed to pH < 8.1 since hatch displaying deformities as puffy carapace (circle), stiff/twisted walking legs (arrow) and lacking antennas (square).

5 Conclusions and future work

There were no clear effects of pCO_2 treatment, on either carapace length or dry weight in *H. gammarus* larvae. However, the high ratio of larvae and juveniles with deformed exoskeletons strongly indicates a negative effect of elevated pCO_2 on European lobster from hatching to one year of age. Some of the deformities may affect the ability to find food and partners (walking legs, claw and antenna), respiration (carapace), and the ability to swim (tail-fan damages). Thus further studies on behaviour and respiration are needed. Studies have already commenced to elucidate mineralization of the exoskeleton in lobster juveniles exposed to various levels of pCO_2 and elevated temperature.

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