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Fertilization success of an arctic sea urchin species, *Strongylocentrotus droebachiensis* (O. F. Müller, 1776) under CO_2-induced ocean acidification

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Abstract

Sea urchins as broadcasting spawners, release their gametes into open water for fertilization, thus being particularly vulnerable to ocean acidification. In this study, we assessed the effects of different pH scenarios on fertilization success of *Strongylocentrotus droebachiensis*, collected at Spitsbergen, Arctic. We achieved acidification by bubbling CO_2 into filtered seawater using partial pressures (pCO_2) of 180, 380, 980, 1400 and 3000 µatm. Untreated filtered seawater was used as control. We recorded fertilization rates and diagnosed morphological aberrations after post-fertilization periods of 1 h and 3 h under different exposure conditions in experiments with and without pre-incubation of the eggs prior to fertilization. In parallel, we conducted measurements of intracellular pH changes using BCECF/AM in unfertilized eggs exposed to a range of acidified seawater. We observed increasing rates of polyspermy in relation to higher seawater pCO_2, which might be due to failures in the formation of the fertilization envelope. In addition, our experiments showed anomalies in fertilized eggs: incomplete lifting-off of the fertilization envelope and blebs of the hyaline layer. Other drastic malformations consisted of constriction, extrusion, vacuolization or degeneration (observed as a gradient from the cortex to the central region of the cell) of the egg cytoplasm, and irregular cell divisions until 2- to 4-cell stages. The intracellular pH (pH_i) decreased significantly from 1400 µatm on. All results indicate a decreasing fertilization success at CO_2 concentrations from 1400 µatm upwards. Exposure time to low pH might be a threatening factor for the cellular buffer capacity, viability, and development after fertilization.

1 Introduction

Ocean acidification (OA) is largely dependent on increasing carbon dioxide (CO_2) emissions into the atmosphere due to anthropogenic activities. The concentration of CO_2 in the atmosphere has a direct effect on the carbonate system of the oceans and thus,

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elevated CO_2 levels modify the distribution of carbonate species (Zeebe and Wolf-Gladrow, 2001), putatively compromising the life of calcifying animal groups. The IPCC-Report (2007) states that by the middle of this century the CO_2 level could reach more than 500 ppm and by 2100 could be over 800 ppm, which means in terms of pH, that the ocean will be 100–150 % more acidic than at the beginning of the industrial revolution (around 280 ppm). Cao and Caldeira (2008) predicted a decrease in pH of 0.25 and 0.48 units when atmospheric CO_2 reaches 550 and 1000 ppm, respectively.

Sea urchins and other broadcasting spawning species might be particularly vulnerable to ocean acidification, especially during critical phases of their life cycle, like fertilization and posterior larval development. Therefore, it is of major relevance to analyze whether gametes released into the acidified environment are protected or not, in order to forecast the potential effects of predicted pH changes on key ecological species. Studies on OA effects on different species have already been done: in fishes (Crim et al., 2011; Melzner et al., 2009; Checkley et al., 2009; Ishimatsu et al., 2004), molluscs (Navarro et al., 2013; Thiyagarajan and Ko, 2012; Dineshram et al., 2012; Parker et al., 2009; Gazeau et al., 2007), crustaceans (Pansch et al., 2012; Li and Gao, 2012; Fitzer et al., 2012; Findlay et al., 2010; Kurihara and Ishimatsu, 2008), corals (Movilla et al., 2012; Fine and Tchernov, 2007; Pelejero et al., 2005) and some sea urchin species (Evans et al., 2013; Albright et al., 2012; Foo et al., 2012; Reuter et al., 2011; Martin et al., 2011; Stumpp et al., 2011b; Dupont et al., 2010; Byrne et al., 2010; Clark et al., 2009; Havenhand et al., 2008).

Sea urchin gametes are widely used models in genomic, embryology and toxicology research (Dupont et al., 2010; Epel et al., 2006; Wong and Wessel, 2005; Wessel et al., 2000; Epel, 1998) and are of high value in studies on development and ocean acidification effects. To our knowledge, there are no studies on effects of OA at the cellular level upon fertilization and this study is the first in identifying the effects of external pH changes, based on CO_2, on intracellular pH (pH_i) of eggs before fertilization. Until now published, results on fertilization experiment using different sea urchins species under acidified conditions are contradictory, mostly due to the variety of experimental

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approaches. The use of different fertilization protocols, limits the comparability of results on OA effects. Therefore, the critical comparison of data should take into account different methodologies and endpoints of effects measured (Reuter et al., 2011).

Moreover, no data exists on the sea urchin species *Strongylocentrotus droebachiensis* from the Arctic. The study of arctic populations offers the possibility to identify effects of OA on reproduction of animals living in a geographic area, where low temperature enhances dissolution of carbon dioxide into seawater. The green sea urchin *S. droebachiensis* is an important key species that stabilizes community structure and is part of established food webs all along its life cycle (Dupont et al., 2010; Addison and Hart, 2004; Scheibling and Hatcher, 2001). *S. droebachiensis* is widely distributed in northern circumpolar waters (Pacific and Atlantic coasts of North America and Arctic Ocean) (Scheibling and Hatcher, 2001; Levitan, 1998a) within a wide range of physically different habitat conditions in terms of temperature and depth. Cyclic environmental changes might influence the acclimation history of animal species within different populations expanding their salinity and pH tolerance. In Kongsfjorden, Svalbard, the seasonal melting cycles produce changes in transparency of water masses and salinity (Svendsen et al., 2002) which may also determine seasonal pH changes due to photosynthetic activity. The spring bloom is the only predictable phytoplankton production peak of the Kongsfjorden (Hop et al., 2002). In Norwest Atlantic, spawning of *S. droebachiensis* takes place in early spring and is triggered by phytoplankton blooms (Scheibling and Hatcher, 2001).

Reproduction traits of this species might be influenced not only by environmental conditions, but also by natural aggregation patterns, reproductive behavior, and gametes longevity in nature (Levitan, 1998a). Meidel and Yund (2001) reported for *S. droebachiensis* an elevated fertilization level held over as much as 48 h in field experiments under natural sperm release events. Release of male gametes in a viscous fluid increases their longevity. These authors also found that under laboratory conditions, viability of the eggs could last even 2–3 days. Thus, we can anticipate that released gametes are exposed to lowered pH conditions for longer periods than experimental

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assays have shown until now, before fertilization take place. Hamaguchi et al. (1997) investigated the (pH_i) response of sea urchin eggs exposed to medium containing weak permeant acid and base diluted into seawater. These authors referred that intracellular pH changed linearly against extracellular pH (pH_o) between 6 and 8 being almost equal to pH_o at certain concentrations of acid and base after 20 min exposure. This response was depending on factors like internal pH before exposure, pH buffering capacity of the cytoplasm and external concentration of passively transported ions. Hence, we can hypothesize that there will be internal pH changes within eggs exposed to OA.

Under these constraints, some general questions are of major relevance, such as whether intracellular pH (pHi) changes due to OA exposure impair fertilization success, whether OA cause detectable morphological and physiological cellular alterations; and which fertilization and developmental phases will be affected under acidified conditions.

Therefore, we focused our study on key events during fertilization and early development. We conducted fertilization experiments in acidified seawater under two approaches: with and without pre-incubation of the eggs before fertilization. We used five levels of acidification obtained by bubbling filtered seawater with different CO_2 concentrations (180, 380, 980, 1400 and 3000 µatm) and untreated filtered seawater as control. In addition, we measured changes in pH_i levels using BCECF/AM in unfertilized eggs exposed to acidified seawater for 1 h.

The objectives of this study are: (i) to analyze whether OA might disturb fertilization and developmental processes like formation of the fertilization envelope (with polyspermy as a lethal endpoint), first cellular divisions, and first larval stages, (ii) to determine whether these effects might be dependent on time of exposure and (iii) to identify if pH_i of spawned eggs changes due to OA.

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2 Materials and methods

2.1 Collection and maintenance of sea urchins

Adult *S. droebachiensis* were collected by SCUBA divers in Kongsfjordneset, Svalbard and transported to seawater aquaria at the Alfred Wegener Institute for Polar and Marine Research (AWI) in Bremerhaven shortly after collection. They were maintained in 250–350 L recirculation aquaria at 2–3 °C temperature controlled laboratories with light regime like their natural habitat at the time of collection for 2–4 weeks before the start of experiments. Each tank was connected to individual EHEIM ecco pro filters. Sea urchins were fed *ad libitum* with algae (*Laminaria* spp. and *Fucus* spp.) or less frequently, with blue mussels (*Mytilus edulis*) maintained in aquarium facilities of the AWI as inclusion of animal protein and carotenes in the diet of *S. droebachiensis* enhances somatic and gonadal growth (Meidel and Scheibling, 1999).

2.2 Experimental set up

In our experiments, we used North Sea seawater (averaged salinity 30.8) filtered through a 0.22 µm Millipore Nitrocellulose membrane (Millipore, Ireland). Seawater was stored in 5 L Duran borosilicate bottles equipped with glass frits for aeration. We manipulated seawater for changing DIC without disturbing alkalinity values, a method which is most appropriate to reproduce the expected situation influenced by atmospheric CO_2 concentrations (Riebesell et al., 2010). Manipulations were conducted at 2.9 ± 0.2 °C air temperature by continuously sparging with humidified filtered air (0.2 µm) of different partial pressures of CO_2 (180, 380, 980 and 1400 µatm) and at 4.9 °C for 3000 µatm. The concentrations of CO_2 were selected according to the literature (Riebesell et al., 2010; IPCC-Report, 2007) to represent atmospheric conditions during the last glacial maximum (180 µatm), present conditions (380 µatm), projected scenarios by 2100 and 2200 respectively (980 and 1400 µatm), and 3000 µatm as an extreme value. As control, we used seawater without CO_2 manipulations, labeled as NB. The gas flow rates

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for 180–1400 µatm set-up were of 130 ± 10 mL min^{-1}. A custom-made gas flow controller generated the gas mixtures by mixing CO_2-free air (< 1 ppm CO_2; Dominick Hunter) with pure Air Liquide CO_2 in a mass flow controller based system (CGM 2000 MCZ Umwelttechnik). In 3000 µatm set-up, we used a fixed gas mixture containing this concentration of CO_2 for sparging. We controlled total alkalinity (A_T) and dissolved inorganic carbon (DIC) in water subsamples taken at the start of each experiment and at the time of sample fixation (description below). We measured A_T with a fully automated titration system (Schott TitroLine Alpha Plus, precision of ±13 µmol kg^{-1}) and DIC with a Technicon TRAACS0 continuous flow analyzer (mean accuracy of ±5 µmol kg^{-1}). Salinity was determined with a WTW Conductivity meter Cond 3110 and an epoxy Standard Conductivity Measuring Cell TetraCon 325/C with integrated temperature sensor. pCO_2 values of control seawater were calculated from A_T/DIC and pH measurements. Seawater was sparged for at least 48–96 h (to ensure equilibration), and stored at 3 °C until use in 1–2 L Duran borosilicate bottles without headspace. Seawater speciation and pH at experimental conditions were calculated with CO2SYS software (Lewis and Wallace, 1998), using dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

2.3 Fertilization experiments

For each fertilization experiment, 4–6 females and 2–4 males were induced to spawn by intracoelomic injection of 1 mL KCl 0.55M. Females were left to shed the eggs into filtered seawater at 2–3 °C while sperm was collected dry from the aboral pores of males and kept in 2 mL Eppendorf Safe-Lock Tubes on ice until use. Sperm quality was assessed by motility and fertilization capability before use and eggs quality by shape, size, presence of visible nucleus and fertilization ability. Eggs were washed in filtered seawater, sieved through mesh screens 40 (425 µm pore) of a Cell Dissociation Sieve-Tissue Grinder Kit (Sigma-Aldrich Co. St Louis, MO USA) and pooled for experiments. Sperm was diluted 2–3 min before the experiments in ASW/FSW to a final dilution

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factor of 10^4 per experimental vial. This concentration was selected based on best rates of fertilization success obtained on this species in relation with sperm to egg contact time (Levitan, 1998b) and according to the sperm cell bioassays conducted by Dinnel et al. (1987).

We let fertilization proceed for 1 h and 3 h (Fertilization time, Ft) with or without previous incubation of the eggs in ASW/FSW. We used these fertilization periods to assess the formation of fertilization envelope (FE) and at least the first cell divisions. Pre-incubation simulates scenarios of asynchronous spawning events also observed in nature, in which gamete contacts either occur immediately, or after some time upon shedding (in our experiments one and three hours Pre- incubation time, PI.t), being exposed to acidified detrimental conditions before fertilization occurs.

Experiments without pre-incubation (WOPI) had three replicates per treatment (pCO_2) and were conducted in experimental 25 mL Duran borosilicate glass Erlenmeyer filled with ASW and closed with a silicon cork. The experiments with pre-incubation (WIPI) of the eggs were done in 100 mL Duran borosilicate bottles closed with silicon septa under the screw cap with three pre-incubation periods (PI.t = 0 h, 1 h, 3 h). After 1 and 3 h Ft, we concentrated the eggs/zygotes in each replicate to 9 mL volume and fixed with 1 mL formalin 10 % in seawater for posterior analysis. We analyzed all fixed samples with 100× magnification under an Olympus BH-2 microscope (Japan) connected to an AxioCam ICc1 coupled to a computer equipped with the software AxioVision (Vers. 4.6.3.0, ZEISS).

In sea urchin fertilization tests, toxicity is indicated by a reduction in the percentage of fertilized eggs when compared to a control sample (USEPA, 2012, 1993). Fertilization is assessed by the presence of a well-developed FE. In our experiments, we classified the eggs taking into account the FE formation and registered other atypical changes in fertilized eggs with respect to the morphology of their FE and hyaline layer (HL). We gave the highest lesion scale to eggs with problems in FE, which implications in polyspermy are well documented in the literature (Reuter et al., 2011; Levitan et al., 2007; Haley and Wessel, 2004; LaFleur et al., 1998; Vacquier et al., 1973). Problems

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in HL followed in relevance because, notwithstanding this layer can be synthesize de novo by the embryos, its defects imply unsuccessful orientation of cells during and after cellular division and might cause embryonal malformations.

We quantified the observed morphologies and made groups according to similarities considering cellular division. The grouped variables were divided into successful fertilization (SF) and unsuccessful fertilization (UF) and used for statistical analysis. Fertilization success was analyzed as the percentage of successful fertilized and unsuccessful fertilized eggs at each treatment. We separately analyzed data from experiments with pre-incubation using the same procedure.

2.4 Early development

We followed the development of the zygotes with eggs batches from two fertilization experiments. Fertilization proceeded as described before and fertilized eggs were left to develop until 24, 48, and 72 h without water exchange at 3 °C. After those endpoints, we collected 1 mL from each treatment vial and took pictures for posterior comparison. Once the ciliated blastula hatched, we fed them daily with 50 µL solution of *Rhodomonas salina*.

2.5 Fluorometric measurements of intracellular pH

We measured intracellular pH changes (pH_i) using BCECF-AM 1mM stock solution in DMSO (Invitrogen Molecular Probes, Oregon USA) as pH indicator diluted in seawater (ASW or FSW) at 5 µM final concentration. We exposed unfertilized eggs obtained from individual females from fertilization experiments mentioned above, to different treatments (180, 380, 980, 1400, and 3000 µatm) for 30 min. Eggs were then incubated for another 30 min in the corresponding ASW including BCECF-AM. Ratiometric measurements of pH changes were conducted using the pH excitation profile of BCECF-AM, which is pH dependent. Fluorescence was obtained through an UV objective (Zeiss NeoFluor 20X) using excitation wavelength of 490 and 439 nm. Emission intensity was

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detected at 535 nm. We measured fluorescence using an imaging system (Visitron Systems, Puchheim) equipped with a CCD camera mounted on an inverted microscope (Zeiss Axiovert 100). About five to six individual cells per sample were measured simultaneously, using the region of interest function of the software (Metafluor, Meta Imaging Series). Five samples were taken for each treatment.

2.6 Statistical analysis

All cells of each replicate were counted and classified according to observed effects. We grouped effects into categories depending on cellular damage and percentage of total in each sample were calculated and used for statistical analysis. Due to the high variability in the water parameters of the NB samples, 380 µatm was used as control in statistical analysis. We conducted exploration statistical analysis and modeling using R (R Development Core Team, 2010) Version 2.12.0.

We applied multivariate generalized linear mixed effects models (GLMM) with logit-link for proportional data, using the package lme4 (Bates, 2012). Since models showed indications of overdispersion, we corrected them by individual-level random effects (Browne et al., 2005). In addition, we included as a random factor, coding for 8 assays in order to account for the same origin of such different clusters of samples.

For statistical analysis of ratiometric measurements, we used IGOR Pro Version 4 and comparison of acidification levels was made using ANOVA and Tukey's HSD test. The level of significance for all tests was $\alpha < 0.05$ and all analyses were two-tailed. Data in the text and figures show mean values ±standard deviation (SD).

3 Results

3.1 Analysis of OA effects on fertilization success

At the acidification treatments used, we observed unexpected effects at cellular level. There were constant water parameters within each experiment. Mea-

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sured water parameters A_T/DIC and calculated pH_T at 3 °C are shown in Table 1 as mean values ±SD. We detected a relation between cellular pathologies and the acidification level used. Table 2 resumes the description of categories into which eggs/zygotes were grouped and the final classification into successful or unsuccessful fertilization (SF-UF).

The proportion of eggs classified as SF or UF in relation to pCO_2 treatments at each experimental approach used is shown in Fig. 3. The frequency of morphological pathologies classified within UF increased significantly for 1400 and 3000 µatm treatments.

A GLMM containing all independent explanatory variables was fitted to the data comprising all experiments together and showed significantly different effects for pH ($\chi^2_1 = 124.72$, $N = 255$, $p < 0.001$), Ft ($\chi^2_1 = 6.92$, $N = 255$, $p = 0.008$), and PI.t ($\chi^2_1 = 15.76$, $N = 255$, $p < 0.001$) on fertilization success (proportion of SF-UF). The variables used in the model (pH, Ft, PI.t) were independent from each other. We used the continuous pH variable in statistical analysis, while pCO_2 was used only as grouping factor in graphics.

Salinity of water sparged with 3000 µatm was higher than the other water set-ups due to higher flow rates during aeration. Therefore it was also included in the model as an independent variable (even when it has a theoretical relation with pH) to verify its effect within the same model and the results showed no significant effect on fertilization success ($\chi^2_1 = 0.01$, $N = 255$, $p = 0.909$).

Figure 4 shows the distribution of the described morphotypes in relation to treatment levels. Analyzing data from all experiments without discerning on experimental approach, we observed that pH changes had clear effects on the proportion of eggs grouped as perfect FE formation (PFE) (which decreases at higher pCO_2 levels) and on the proportion of polyspermic eggs with no FE formation (NFE) (that increases at higher pCO_2 levels). pH changes seems not to be related to the proportion of eggs within hyaline blebbing (HB) and partial lifting-off of FE (PLO). Although eggs grouped within not-fertilized (NFert) and polyspermic eggs with FE formation (WFE) showed no marked

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relation to pCO_2 treatments, its proportion slightly increased from non-acidic to acidic conditions, except in 3000 µatm treatments for which the proportion of WFE decreases.

There was a slightly increase in the pool of successfully fertilized eggs (SF) in relation to the time course of fertilization (from Ft 1 h to 3 h). Post-hoc analysis of fertilization time effects showed that there were significantly differences between normal (380 µatm) and acidic (1400/3000 µatm) conditions ($\chi^2_1 = 5.54$, $N = 89$, $p = 0.019$; $\chi^2_1 = 8.29$; $N = 76$; $p = 0.004$, respectively) independently of the approach used.

Data from experiment with pre-incubation (WIPI) revealed significant effects of pH ($\chi^2_1 = 58.90$, $N = 109$, $p < 0.001$) and PI.t ($\chi^2_1 = 16.44$, $N = 109$, $p < 0.001$) on fertilization success, while Ft had no significant effects ($\chi^2_1 = 1.72$, $N = 109$, $p = 0.189$). Post-hoc analysis of WIPI experiments showed significantly differences between 380 µatm and 1400/3000 µatm treatments ($\chi^2_1 = 14.65$, $N = 38$, $p < 0.001$; $\chi^2_1 = 42.09$, $N = 37$, $p < 0.001$, respectively).

3.2 Evaluation of OA effects on early development

The samples taken to follow up the development of the zygote showed a pH/pCO_2 dependent increase in malformation rates and a delay in development, which slowed down at 1400 µatm and was delayed or even stopped at 3000 µatm.

On samples collected at 24 h we observed mostly zygotes in early to mid blastula at 180, 380, 980 µatm and NB. For 980 and 1400 µatm there was an increment in malformations but most of the zygotes in 1400 µatm were at the beginning of early blastula. In 3000 µatm there were also malformations and an elevated rate of not-divided cells and zygotes in a stage before blastula (Fig. 5a).

At 48 h an elevated proportion of perfectly hatched ciliated blastula were observed at 180, 380, 980 µatm and NB and zygotes started to rotate (Fig. 5b). A small proportion of hatched blastula with rotation movements were also found in 1400 µatm but in 3000 µatm no movements were observed and the majority of the cells did not succeed the first divisions.

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Zygote developments at 72 h, were at early, middle or late gastrula depending on treatment. Especially 180, 380 and 980 µatm treatments showed gastrula with projection and contact of the archenteron with the animal pole region (Fig. 5c). Gastrulae from control without CO_2 bubbling (NB) were already at the beginning of invagination of the vegetal plate to produce the archenteron, similarly to 1400 µatm treatments. There were differences between replicates of 1400 µatm probably due to lost of the desired CO_2 partial pressure, and it was found that some zygotes were at ciliated mesenchyme blastula and early gastrula with restricted movement patterns while others were totally developed as in non-acidic conditions (Fig. 5d). 3000 µatm treatment had mostly no further development but elevated proportion of irregular divisions and degeneration of dead zygotes.

3.3 Intracellular pH (pH_i) under exposure to CO_2-induced OA

Figure 6 refers the results of fluorescence ratio 490/439 in pre-incubated/not fertilized eggs using BCECF-AM as pH indicator. Measurements after exposure to different acidification levels (180–3000 µatm) showed significantly different ratios of 1.51 for 1400 µatm 3000 µatm when compared with ratios measured for 180, 980 and 380 µatm (1.62, 1.67 and 1.68, respectively) (ANOVA $F_{4,115} = 17.9$, $N = 24$, $p < 0.001$). Precise intracellular pH values were not calculated because of logistic problems due to experimental design (calibration at the same day of the measurements were not possible). Nevertheless, clear changes in pH_i demonstrated by different fluorescence ratios, are shown for 1400 and 3000 µatm.

4 Discussion

Our findings demonstrated significant impacts of OA on fertilization success of *S. droebachiensis* eggs at the cellular level at elevated seawater pCO_2 concentrations (1400 µatm and 3000 µatm). In addition, OA has effects also on the first cell divisions,

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intracellular pH and buffer capacity of exposed unfertilized eggs. Recent studies describe developmental delays and impacts of OA on survival, calcification and growth patterns of larvae from different sea urchin species (Evans et al., 2013; Stumpp et al., 2011a,b; Sheppard Brennand et al., 2010). In our study, we showed that the detrimental effects of OA start as soon as the eggs are shed and in contact to acidic conditions.

4.1 OA effects on fertilization success

Successful fertilization is currently solely assessed by the presence of a well-formed and elevated fertilization envelope surrounding a fertilized egg and its hyaline layer. Our morphological evaluation evidenced that, analysis of fertilization success tests according to the presently published standard protocols (USEPA, 2012, 1993) appear to be insufficient for the assessment of OA effects and should also include alterations of cellular structures other than fertilization envelope.

To our knowledge, the literature about effects of pH on HL and FE formation in the context of cell biology is scarce. Based on our experiments we conclude that at acidification levels of 1400 µatm and 3000 µatm, the successful formation of fertilization envelope is significantly impaired, and that under CO_2-induced OA conditions pathological phenomena, e.g. partial lifting-off (PLO), hyaline blebbing (HB), and increasing polyspermy rates emerge.

We detected polyspermic eggs, which already had fertilization envelope (WFE) with no marked dependence to pCO_2 treatments used, but that slightly increased until 1400 µatm and decreased at 3000 µatm. It might be possible that a proportion of the artificially shed eggs were also affected by other factor than pCO_2, e.g. sperm : egg ratio or gamete quality features which were not detected by gamete examination before the assay. We did not include sperm : egg ratio in the analysis of results. The evaluated data in this study is divided as proportions from a total count, thus the decrease in the proportion of polyspermic eggs with FE formation (WFE) observed at 3000 µatm is reflected probably in an increase in not fertilized (NFert) and polyspermic eggs with no FE formation (NFE) within this treatment.

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Reproduction in nature is ruled by other sperm : egg ratios than those used in fertilization experiments, being too low to render polyspermy. Disturbances in the timing of FE formation and cell activation after fertilization might be enhanced by OA. Once the metabolic activation takes place, the FE formation should already be accomplished. Under laboratory conditions and exposition to OA, in which the FE formation is impared, slowed down, or even not totally completed at the time of metabolic activation, supernumerary sperms could increase the probability of polyspermy events. Dale and DeFelice (2011) proposed that monospermy under natural conditions, is ensured by the controlled and gradual encounter of a minimum number of spermatozoa with the oocyte and that fine tuning is ensured by the structural and molecular organization of the oocyte and its surrounding coat.

Eggs grouped in the partial lifting-off of FE (PLO) category had a similar independent response to pCO_2 exposure but a high variability in morphotypes. A small proportion of eggs within this category accomplished the first cell division while being attached to the partially lifted FE.

Stephens (1972) found for the same species we used in our experiments, that FE is completed after 7 min at 4 °C and 10 min at 0 °C. In our experiments at 3 °C, the FE formation proceeded longer than 10 min in not treated FSW and in ASW.

There are few studies describing the phenomenon of blebbing of FE and HL together. Among the few studies the work of Epel (1975) describes that improper elevation of fertilization envelope in the presence of trypsin inhibitors rends eggs that looks like "bubbly rosettes" and he suggests that the vitelline layer (precursor from FE) is attached to the plasma membrane by protease-sensitive bonds.

Epel (1975) also mentioned that there are two kinds of protease within the cortical granules, which are released upon fertilization and are involved in different processes: detachment of the vitelline layer from plasma membrane and detachment of supernumerary sperm from the still not hardened FE.

Other studies identified and characterized the proteins involved in the above process (Wong and Wessel, 2008; Haley and Wessel, 2004a, b, 1999) and observed fertilized

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eggs with similar "blebby" envelopes to those obtained in our study, after been treated in artificial seawater adjusted to pH 8, 7, 6, 5 and 4. Haley and Wessel (2004b) showed that the Cortical Granules Serine Protease 1 (CGSP1), a protein secreted during the cortical reaction, is inhibited at lowered pH, decreasing its activity down to 25 % at pH 7. CGSP1 is activated at fertilization, mediates the protease-responses that follow the cortical reaction and contribute to the block to polyspermy.

In our study, the proportion of eggs classified as polyspermic with no FE formation (NFE) increased at high pCO_2 values. It might be possible that in our experiments the FE formation could be affected by a similar mechanism, e.g. inhibition of CGSP1 activity. At acidic conditions, the FE is often detached from the egg, and we found FEs in the medium with sperm still attached at its surface. The presence of detached FE in experimental vials might indicate that pH changes due to the solution of CO_2 into seawater could destabilize the FE and impair the spermicidal function of the proteases involved in the detachment of supernumerary sperms, thus potentiating the risk of polyspermy.

We observed defects in the formation of the HL at all OA levels used, which might also compromise to some extend the embryonic development, but these were not dose-related to the experimental changes in pCO_2 and might be probably driven by internal responses to exposure or fixation, thus changing from egg to egg. We included eggs showing defects in HL formation within the classification of successful fertilization because cellular divisions were found to occur. Nevertheless, blebbing was diverse (supplementary material) and could be also observed in eggs classified as polyspermic with FE formation (WFE) under acidic pCO_2 levels. The HL is referred to be firmly attached to the embryo; it is not lost until metamorphosis of the larvae and becomes difficult to observe when it is partially digested or weakened (Citkowitz, 1971). Stephens (1972) stated that HL in *S. droebachiensis* is completely formed after 120 min at 4 °C. Yet, in our experiments, firmly attached HLs were only observed in eggs grouped within perfect fertilization envelope (PFE) after 1 h, while after 3 h the proportion of eggs with slightly separated HL increased. HL blistering patterns diverge from totally separated from the cell surface to rosette-like structures at a different extent. In general, blebbing

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increased with fertilization time independently of which experimental approach was used and this pathology was observed also in dividing eggs.

Studies on hyaline focused on its role during embryonic development as substrate for cell adhesion (Wessel et al., 1998), and during the gastrulation processes (Citkowitz, 1971). There is not much information about its role in the time lapse between fertilization and first division, but it is supposed to keep the cells in the right orientation for division.

The eggs with perfect FE formation (PFE) represents the proportion of eggs with good quality and fertilization capacity. This group, compared with not fertilized (NFert), would be used in current fertilization tests to assess toxicity. pH had significant effects on the frequency of PFE in our experiments for acidic treatments, especially in pre-incubation experiments. This finding indicates a deterioration of the eggs due to pre-incubation procedures and an increasing inability of the eggs to cope with pH changes after longer exposure periods.

Within the published literature on OA effects using fertilization tests, different methodological approaches and different species have been used and responses are variable and even contradictory: sea urchins seems either to be robust to OA manipulation experiments (Martin et al., 2011; Byrne et al., 2010, 2009), or sensitive to OA (Reuter et al., 2011; Stumpp et al., 2011a; Havenhand et al., 2008; Kurihara et al., 2004; Kurihara and Shirayama, 2004).

Analysis of the natural habitat conditions prior to experimental manipulations might help to assess possible influencing factors, such as pre-adaptation, that could also explain, at least to some extent, differences in population and species-specific responses to acidification.

Water temperature and salinity influence the solubility of CO_2 in seawater and pH typically decreases with depth in the oceans, particularly in oxygen minimum zones (Riebesell et al., 2010). Alkalinity might be influenced by evaporation and recirculation processes and there are several studies about habitat pH variability in the literature. Jokiel et al. (2008) measured diurnal cycles in solar radiation, temperature and

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seawater chemistry (pCO_2, pH, A_T and aragonite saturation state) in a mesocosm study on corals. Pelejero et al. (2005) found a clear interdecadal oscillation of pH with a cyclicity of about 50 yr, synchronous with the Interdecadal Pacific Oscillation, which could give an insight into possible mechanisms driving long-term pH variations in seawater. Upwelling waters might also be involved in changes in pH (Pelejero et al., 2005). A detailed analysis on environmental conditions (depth, seasonal hydrography, circulation patterns, climatic factors, long-term patterns on community and ecosystem states, as well as anthropogenic activities), could bring more light into possible pre-adaption processes and support for interpretation and comparison of experimental results conducted with different species collected at specific habitats.

Most of the published data showing no effect of OA on fertilization success were conducted using species from tropical and subtropical areas, mostly exposed during its entire life cycle to variable conditions in relation to pH, light regime, and temperature. Independently from methodological procedures used, the natural habitat variations have an influence on the stress responses of the study organisms. The study of Evans et al. (2013) using as model *Strongylocentrotus purpuratus* from up-welling zones in the North Pacific, evidenced that species naturally exposed to acidified conditions become tolerant to low and variable pH. These authors analyzed transcriptomic responses of early stage embryos and larvae exposed to acidified conditions and found that compensation can occur for important processes like calcification, even at early life stages, and at metabollically expensive costs in terms of energy. Similar results on compensatory responses were found for *S. droebachiensis* (Stumpp et al., 2011a, b) collected from populations in Kattegat which are already exposed to seasonally elevated pCO_2 and hypoxia.

Stumpp et al. (2011a) reported that impact of OA manipulations on sea urchin larvae cause developmental delay, elevated metabolic rates and changes in gene expression patterns.

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In our study, the observed effects in pre-incubation experiments could also be related to the progressive impairment of the buffer capacity of the cells that had been exposed for longer periods to lowered pH conditions.

4.2 OA effects on early development

We observed a delay in the process of cell division in relation to the concentrations of CO_2 used. This process is reversible, as revealed by the loss of the requested partial pressure in some replicates of 1400 µatm treatment, in which zygotes fully developed into healthy larvae. A developmental delay could be also the consequence of changes in the internal pH and buffer capacity of the eggs. Relative small changes in intracellular pH could act as control mechanism in regulating the assembly of actin filaments, as demonstrated in isolated sea urchin cortices showing different patterns of actin polymerization at different pH treatments (Begg and Rehbun, 1979). Actin and tubulin are actively involved in the process of activation of metabolism and cellular division, playing a key role in excision and separation of daughter cells, segregation of organelles and in cell to cell interactions within the zygote. Movements of organelles and molecules are also mediated by cytoskeletal structures, which dynamic might be threatened by exposure to acidification.

For *S. droebachiensis* individuals, collected at Cape Cod Bay, the first division at 4 °C is mentioned to occur after 5 h (Stephens, 1972). In our experiments, at 3 °C, there was a small proportion of divided eggs after 3 h, but hatching took until 48 h under non-acidic conditions similarly to values referred by Stephens (1972). He suggested that the duration of the entire developmental program until hatching seems to be time-regulated depending on temperature. At many pivotal points, the fertilization process depends on the fine regulation of actin filaments: during gamete fusion, (that occurs in sea urchin selectively at the microvilli regions of the egg surface, which are actin-rich); sperm penetration (sperm-borne actin rod appears to be physically connected with the actin cytoskeleton in the egg cortex), cortical granules exocytosis, and intracellular Ca^{2+} release (Chun and Santella, 2009). Microvilli elongation during gamete fusion is due to

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actin filament bundle formation and this is triggered by an increase in cytoplasmic pH at fertilization (Begg et al., 1982). Using isolated cortices of unfertilized and fertilized sea urchin eggs, Begg and Rehbun (1979) showed that pH regulates the polymerization of actin (at pH of 7.3 or above). These authors demonstrated that the state of actin can be experimentally controlled by manipulating the pH in the media

In order to assess the underlying mechanism of CO_2-mediated pH changes within the oocytes and zygotes and its implications in the observed developmental delay, we presently conduct analysis of the cytoskeletal integrity and function in the frame of our OA studies.

4.3 OA effects on pH_i of pre-incubated eggs

Finely tuned internal pH modifications are important for the activation of the cells after fertilization. Therefore, the limited buffer capacity of a cell stressed by external acidified conditions might influence its ability to respond to activation mechanisms involved in fertilization and first divisions. Cells are able to compensate for these effects, but at expenses of energy, which should have been allocated to other vital processes like division and development. Our results showed that until pH values around 7.8 (in 980 µatm treatment), the eggs of *S. droebachiensis* are able to compensate the detrimental effects of extracellular pH changes due to CO_2. Elevated concentrations of CO_2 into seawater, e.g. 1400 and 3000 µatm, are associated to pH_i levels, which overstrain the compensatory capacities of the eggs and under these conditions expensive compensation processes might compromise the energy budget for posterior development.

Using medium containing acetic acid and ammonium, Hamaguchi et al. (1997) found that pH_i can be regulated in a wide range of pH around the original pH_i value. After 10–20 min treatment in acidic and basic conditions, pH_i was found to coincide with pH of the external medium due to passive ionic transport. However, these authors used weak bases and acids, for which the mechanisms for regulation of pH_i are entirely different from CO_2-related acidification.

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Evans et al. (2013) suggested that intracellular compensation might involve transmembrane movements of protons (H^+) and bicarbonate (HCO_3^-) via Na^+/H^+ and Cl^-/HCO_3^- exchangers using energy. These authors showed that the transcription of the related genes for these mechanisms did not change in *S. purpuratus* larvae under exposure to acidic conditions, probably due to pre-adaption.

4.4 Summary and future perspectives

We conducted fertilization experiments with parallel measurements of pH_i in unfertilized eggs and tracking of the further development to determine possible cellular effects of simulated OA exposure. We examined the putative effect of time of exposure to acidic conditions on fertilization success. Our data indicate that fertilization success of broadcasting spawner, like sea urchins, might be threatened by acidic conditions expected to occur for 2100 on. OA impact might be stronger than expected when we consider the percentage of eggs with cellular pathologies in our fertilization tests. Stressed animals exposed to acidified conditions might produce less gametes, and once these are shed, a high percentage will be damaged by an unfavorable acidic environment. Pre-adaption might help to palliate OA impact in some population, but exposed animals at stable environments, like the Arctic ocean, could face bottleneck effects. Especially for species which natural habitat does not promote pre-adaption strategies, the knowledge of mechanisms involved in reproduction and survival should be a core issue in order to forecast OA impact at higher organization levels. Research, focused on the cellular level should be implemented in the frame of future OA risk assessment studies. These must include measurements of pH_i and direct observations of effects on cytoskeleton and organelle functions of exposed eggs upon fertilization and cell division. We analyzed the effects of pH as a single variable, but other environmental factors like temperature, salinity, hypoxic conditions, chemical pollution and biological reproductive traits (e.g. gonadal development, reproductive behavior, rates of gamete production and state of the population) of the species under study should be incorporated in future approaches. Comparisons of different species responses under a standardized

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protocol for fertilization test in the frame of OA should be employed to elucidate differences in results already published.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/10/8027/2013/bgd-10-8027-2013-supplement.pdf.

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Table 1. Seawater parameters (mean ± SD) of each treatment (NB, 180, 380, 980, 1400 and 3000 µatm) used in fertilization experiments. In each experiment, Salinity/A_T/DIC values were calculated as a mean of two/three measurements per sample and experiment. pH_T calculations were made assuming equal input conditions for A_T and DIC measurements of 20 °C, 1 dbar pressure, 0 values for phosphate and silicate.

	Fertilization experiment treatments											
	NB ~ 380 µatm		180 µatm		380 µatm		980 µatm		1400 µatm		3000 µatm	
Measured		*n*		*n*		*n*		*n*		*n*		*n*
Salinity	30.68 ± 0.52	44	30.74 ± 0.44	45	30.75 ± 0.49	44	30.7 ± 0.5	44	30. 87 ± 0.44	44	31.08 ± 0.86	33
A_T (µmol kg^{-1} SW)	2412.73 ± 26.32	44	2401.34 ± 16.85	45	2387.86 ± 20.38	44	2389.57 ± 16.25	44	2389.76 ± 15.19	44	2430.09 ± 21.89	33
DIC (µmol kg^{-1} SW)	2263.8 ± 44.6	44	2162.82 ± 40.18	45	2261.09 ± 13.52	44	2332.44 ± 22.25	44	2396.13 ± 37.52	44	2505.35 ± 24.42	33
Calculated for 3 °C												
pH_T	8.11 ± 0.06	44	8.31 ± 0.08	45	8.13 ± 0.14	44	7.84 ± 0.07	44	7.63 ± 0.14	44	7.40 ± 0.09	33

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Table 2. Categories diagnosed and eggs classification for assessment of fertilization success.

Category	Description	Classification
Perfect Fertilization Envelope formation (PFE) Fig. 1a–d	Eggs in a central position with a well-formed FE around. The HL was visible or not, tightly in contact with the eggs surface or slightly separated from it. Cellular division proceeded until 2-cell or 4-cell stages.	SF
Partial Lifting-Off of FE (PLO) Fig. 1g–i	Eggs/zygotes showing problems in the lifting-off of the FE and which HL, when visible, attachs to the FE. Variations in the degrees of PLO included FE sticked to one or many points (in a rosette-like structure) of the egg surface. Cellular division proceeded until 2-cell or 4-cell stages.	SF
Hyaline Blebbing (HB) Fig. 1e–f and supplementary material	HL of eggs/zygotes blebbing around the eggs surface, within a perfectly formed FE. HL blebs differed in the degree of blister separation from the egg, forming a "rosette-like" structure within the perfectly formed FE.	SF
Polyspermic eggs with FE formation (WFE) Fig. 2d–i	a. Eggs with FE well formed or partially lifted, showing evidence of polyspermy (seen as a great amount of sperms within FE and HL). HL blebbing might be observed. b. Eggs with FE well formed or partially lifted, which divided irregularly until the 2-cell stages. Hyaline blebbing might be observed. c. Eggs showing deformation (with cytoplasmic constriction/extrusion), vacuolization and degeneration (detected as a gradual density change in cytoplasm with a gradient from the cortex to the central region of the cell) within a well or partially formed FE.	UF
Polyspermic eggs with No FE formation (NFE) Fig. 2b–c and supplementary material	a. Fertilized eggs which FE was not lifted-off (observed as a tight halo around the zygote), or was lost (re-found in incubation medium as an empty envelope). b. Fertilized eggs irregularly divided until the 2-cell stage, without FE. c. Eggs without FE showing deformation (with cytoplasmic constriction/extrusion), vacuolization, and degeneration of the cytoplasm.	UF
Eggs Not Fertilized (NFert) Fig. 2a	Rounded eggs with no sign of FE formation or HL.	UF

FE: Fertilization envelope;
HL: Hyaline layer; SF: Successful fertilization;
UF: Unsuccessful fertilization

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Table 3. Nomenclature.

FE:	fertilization envelope;
HL:	hyaline layer;
Ft:	fertilization time;
PI.t:	pre-incubation time;
pH_i:	intracellular pH;
SF:	successful fertilization;
UF:	unsuccessful fertilization.

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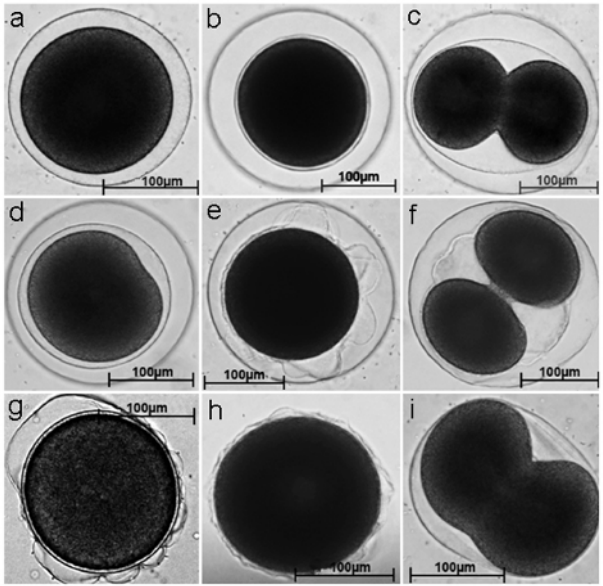

Fig. 1. Successful fertilization in *Strongylocentrotus droebachiensis*. **(a–d)** represent the common states of eggs/zygotes grouped within "Perfect Fertilization Envelope formation (PFE)"; **(e–f)** show two of the features included in "Hyaline Blebbing (HB)" and **(g–i)** illustrate eggs/zygotes belonging to the category "Partial Lifting-Off of FE (PLO)". 100× magnification used.

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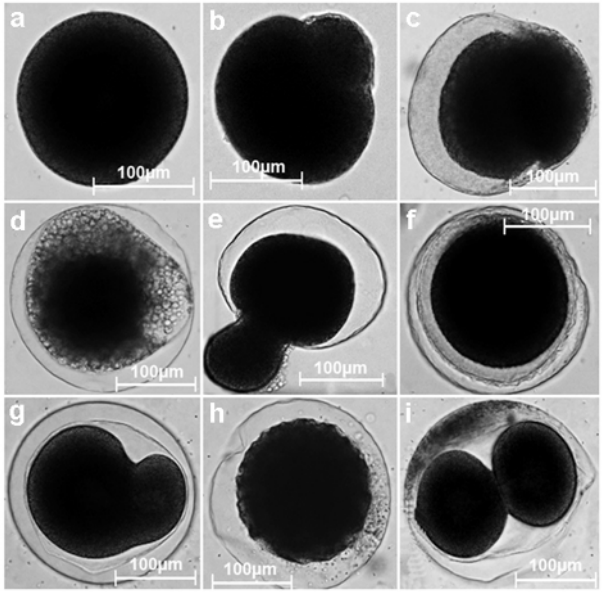

Fig. 2. Unsuccessful fertilization in *Strongylocentrotus droebachiensis*. **(a)** represents the eggs grouped in the category "Not Fertilized (NFert)"; **(b–c)** eggs/zygotes classified as "Polyspermic with No FE formation (NFE)"; **(d–i)** illustrate the aberrations included in "Polyspermic with FE formation (WFE)". Note polyspermic eggs/zygotes showing sperms within the FE in **(h–i)**. 100× magnification used.

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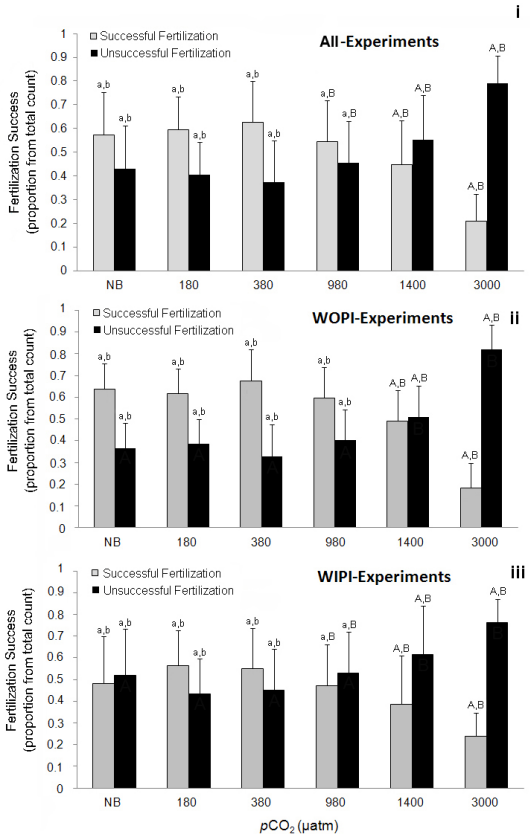

Fig. 3. (i) Fertilization success of *Strongylocentrotus droebachiensis* (SF, grey bars and UF, black bars) at six different pCO_2 treatment. Values are expressed as mean ±SD and capital letters indicate statistically significant differences between pH-pCO_2 (a/A, $p < 0.001$) and Ft-PI.t (b/B, $p < 0.01$) levels (post-hoc pairwise comparison, $N = 255$). **(ii)** (WOPI) without pre-incubation approach ($N = 146$). **(iii)** (WIPI) with pre-incubation approach ($N = 109$).

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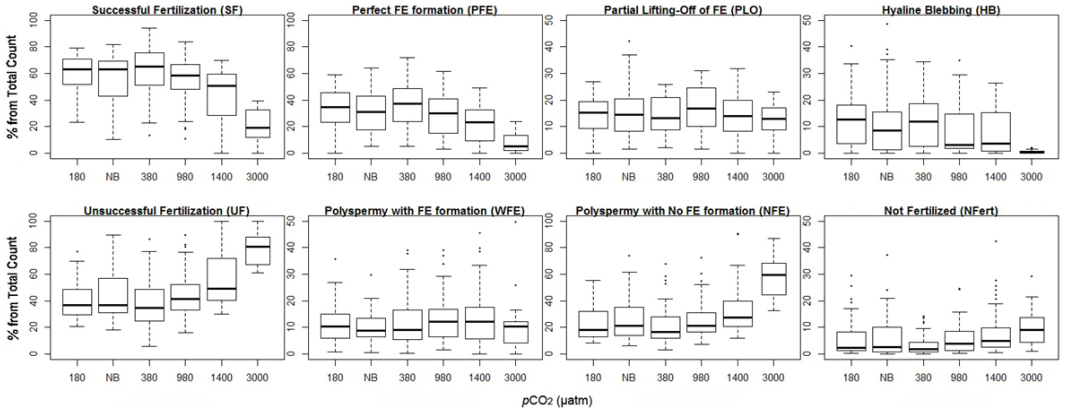

Fig. 4. Proportion of eggs within each category (as percentage from total count) used for assessment of fertilization success (SF-UF) plotted against pCO_2 treatments. (Supplementary material shows the variability in appearance of eggs classified within NLO and HB, 100× magnification used).

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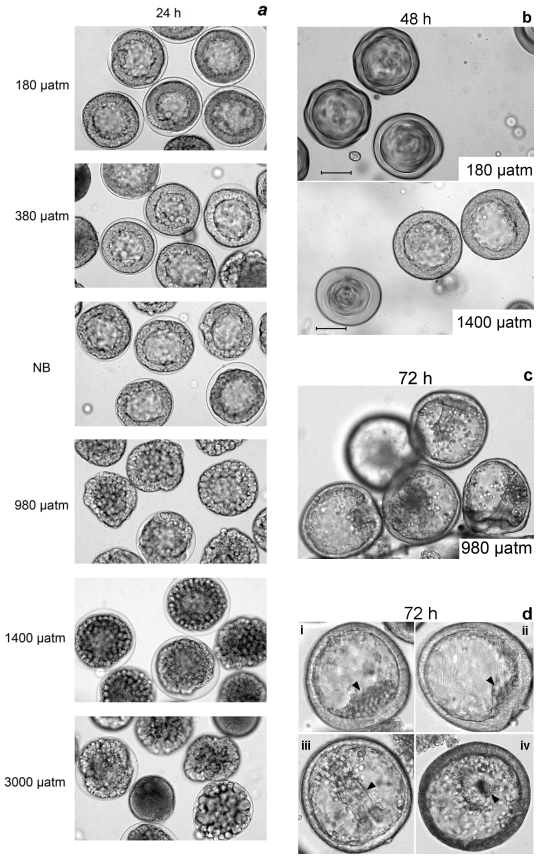

Fig. 5. (a) Impact of CO_2-simulated OA on the development of *Strongylocentrotus droebachiensis,* (24 h post-fertilization) under six pCO_2 treatments (NB-3000 µatm). **(b)** Details of zygote movements at 180 and 1400 µatm treatments after 48 h. **(c)** Normal development after 72 h under 980 µatm. **(d)** Morphological details of archenteron (arrowheads) in delayed development at 1400 µatm **(i–ii)** vs. normal development after water equilibration **(iii–iv)**.

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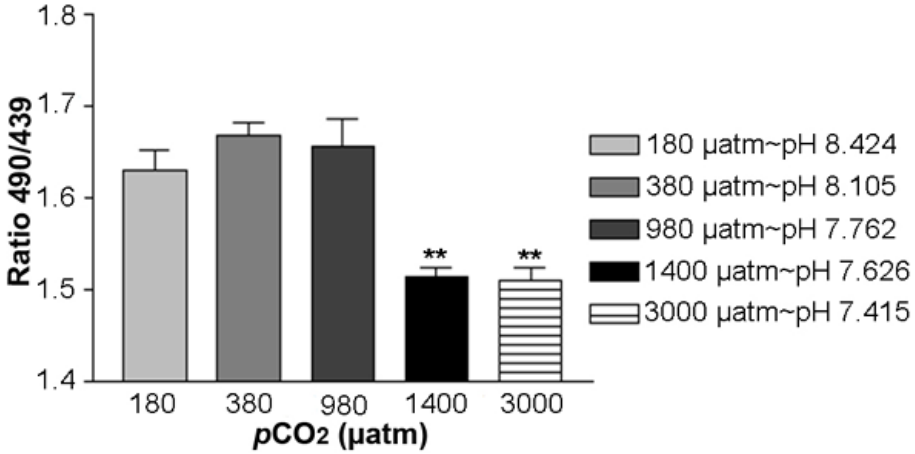

Fig. 6. Fluorometric measurements of pH_i changes in unfertilized sea urchin eggs of *Strongylocentrotus droebachiensis* due to exposure to CO_2-simulated OA. The figure shows fluorescence ratios 490/439 plotted against pCO_2 treatments. The legend indicates pCO_2 levels used and the related pH_e. Statistical significance were observed between treatments used (ANOVA, $F_{4,115} = 17.9$, $N = 24$, $p < 0.001$, (**) denotes significantly differences in post-hoc Tukey HSD test).

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