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ORIGINAL ARTICLE

CO_2-induced fertilization impairment in *Strongylocentrotus droebachiensis* collected in the Arctic

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Abstract Fertilization depends on distribution and aggregation patterns of sea urchins which influence gamete contact time and may potentially enhance their vulnerability to ocean acidification. In this study, we conducted fertilization experiments to assess the effects of selected pH scenarios on fertilization success of *Strongylocentrotus droebachiensis*, from Spitsbergen, Arctic. Acidification was achieved by aerating seawater with different CO_2 partial pressures to represent pre-industrial and present conditions (measured ~180–425 µatm) and future acidification scenarios (~550–800, ~1,300, ~2,000 µatm). Fertilization success was defined as the proportion of successful/unsuccessful fertilizations per treatment; eggs were classified according to features of their fertilization envelope (FE), hyaline layer (HL) and achievement of cellular division. The diagnostic findings of specific pathological aberrations were described in detail. We additionally measured intracellular pH changes in unfertilized eggs exposed for 1 h to selected acidification treatments using BCECF/AM. We conclude that (a) acidified conditions increase the proportion of eggs that failed fertilization, (b) acidification may increase the risk of polyspermy due to failures in the FE formation supported by the occasional observation of multiple sperms in the perivitelline space and (c) irregular formation of the embryo may arise due to impaired formation of the HL. The decrease in fertilization success could be also related to the observed changes in intracellular pH at pCO_2 ~ 1,000 µatm or higher.

Keywords Hyaline layer · Polyspermy · Intracellular pH · Hyaline blebs · Ocean acidification

Abbreviations

OA	Ocean acidification
UnF	Unfertilized
F	Fertilized
FSW	Filtered seawater
ASW	Acidified seawater
FE	Fertilization envelope
PFE	Perfect FE
HL	Hyaline layer
HB	Hyaline blebs
PLO	Partial lifting-off of the FE
NLO	No lifting-off of the FE
FF	Fertilization failure
PVS	Perivitelline space
Ft	Fertilization time
pH_i	Intracellular pH
Fertilization success	(SF: successful fertilization, UF: unsuccessful fertilization)

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Introduction

Ocean acidification (OA) is an emerging problem, nowadays largely originated by increasing carbon dioxide (CO_2) emissions into the atmosphere due to fossil fuel

combustion (Le Quere et al. 2009). Cao and Caldeira (2008) predicted a decrease in pH of 0.25 and 0.48 units when atmospheric CO_2 reaches 550 and 1,000 ppm, respectively. At regional scale, the study of Arctic populations offers the possibility to identify effects of OA on species living in a geographic area where low temperature enhances carbon dioxide dissolution in seawater. This region is already naturally low in calcium and carbonate ion concentration (Orr et al. 2005). It is expected that high-latitude surface waters will be at first affected by a considerable aragonite under saturation and pH changes (Steinacher et al. 2009) and under saturation for the calcite form of calcium carbonate is expected to follow (Orr et al. 2005). This might have adverse consequences for calcifying organism at these latitudes.

Sea urchins are of common use in studies on fertilization and development (Matranga 2005) and because of the reliability of fertilization assays as screening test for environmental monitoring, sea urchins have been frequently used in genomic, embryology, toxicology and ocean acidification research (Epel 1998; Wessel et al. 2000; Wong and Wessel 2005; Epel et al. 2006; Dupont et al. 2010). Previous studies have shown that the life of calcifying groups may be compromised due to OA (Orr et al. 2005; Li and Gao 2012; Movilla et al. 2012; Navarro et al. 2013). It has been demonstrated that adult sea urchins are able to minimize the acidification effect on their skeleton by increasing the concentration of bicarbonate within their perivisceral coelomic fluid and preventing their loss throughout the intestine (Holtmann et al. 2013; Stumpp et al. 2012b). These authors stated that calcification and growth under increased pCO_2 conditions can still proceed, even if at a reduced rate. Yet, the spines will become fragile in an acidic environment probably affecting their resistance to predation with still unknown consequences for population stability.

Vulnerability to OA is expected to be critical at initial phases of organisms' life cycles (Stumpp et al. 2013). Many studies have previously focused on the effects of OA at different life stages on a variety of sea urchin species and have reported a wide range of responses to changes in pCO_2 (Byrne et al. 2010; Martin et al. 2011; Stumpp et al. 2011b; Albright et al. 2012; Foo et al. 2012; Evans et al. 2013; Reuter et al. 2011). Reuter et al. (2011) stated that the observed variability in sea urchin fertilization results may be attributed to different methodologies. On the other hand, different experimental routines lead to the analysis of OA effects in synergy with other important and intrinsically related factors. In our study, we added the effects of time of exposure linked to the fertilization envelope (FE) formation as a crucial parameter to be taken into account.

We choose the green sea urchin *Strongylocentrotus droebachiensis* for our study due to its importance as key species being part of established food webs all along its life cycle (Scheibling and Hatcher 2001; Addison and Hart 2004; Dupont et al. 2010). It is widely distributed in northern circumpolar waters (Pacific and Atlantic coasts of North America and Arctic Ocean) (Levitan 1998b; Scheibling and Hatcher 2001) within a wide range of physically different habitat conditions in terms of temperature and depth. *S. droebachiensis* have already been used in other OA studies (Dupont et al. 2013; Stumpp et al. 2012b; Dupont and Thorndyke 2012; Spicer et al. 2011; Siikavuopio et al. 2007), but information from Arctic populations is still scarce, and to our knowledge, no data exist on fertilization success under increased pCO_2 conditions for this species. Moreover, the species has already been listed as a northern species, which may either decrease in abundance or disappear from northern Britain due to climatic changes (Hiscock et al. 2004) making the evaluation of its robustness to ocean acidification a subject of a great interest.

The population chosen for our study inhabits shallow waters near Ny-Ålesund (Kongsfjorden, Svalbard, Spitsbergen) but can be found between 10 m (own collection data) and 200 m depth (Laudien and Orchard 2012). In addition, it is adapted to the extreme winter temperatures and high salinity variations during summer (Svendsen et al. 2002).

The reproduction traits of this species might be influenced by environmental conditions and natural aggregation patterns affecting the gametes longevity in nature (Levitan 1998a). Meidel and Yund (2001) reported for *S. droebachiensis* an increased fertilization level maintained over 48 h in field experiments under natural sperm release events. These authors also found that under laboratory conditions, eggs could even be fertilized 2–3 days after being shed. Therefore, we anticipate that eggs spawned in the field may be exposed to OA for longer periods than experimental assays have shown until now, before fertilization takes place.

There are references for quite a few sea urchin species providing intracellular pH (pH_i) values before and after fertilization (Table 1). To our knowledge, no data have yet been published reporting pH_i of *S. droebachiensis* eggs. Hamaguchi et al. (1997) observed that pH_i changed linearly against extracellular pH (pH_o) between 6 and 8 and that pH_i was almost equal to pH_o at certain concentrations of acid and base in the media after 20-min exposure. This response depended on pH_i before exposure, pH buffering capacity of the cytoplasm and external concentrations of passively transported ions. This addressed our interest to determine whether eggs exposed to OA would react either by dropping their pH_i or if otherwise, they are able to regulate and maintain their original pH_i values.

To our knowledge, there are no studies on CO_2-induced OA effects on the morphology of the eggs after

Table 1 Overview of the intracellular pH in unfertilized (UnF) and fertilized (F) eggs of different sea urchin species

Species	pH_i (UnF)	pH_i (F)	Method	References
Lytechinus pictus	6.84	7.3	BCECF–dextran	Shen and Buck (1993)
	7.22	7.58	BCECF–dextran	Rees et al. (1995)
	6.90	7.17	DMO method	Dubé et al. (1985)
Paracentrotus lividus	7.38	7.64	DMO method	Payan et al. (1983)
Strongylocentrotus purpuratus	7.08	7.47	DMO method	Johnson and Epel (1981)
	7–7.09	7.54–7.50	DMO method	Dubé et al. (1985)
Schaphechinus mirabilis	6.8	7.34	Microfluorometry using pyranine	Hamaguchi et al. (1997)
Hemicentrotus pulcherrimus	6.81	7.32		
Psammechinus miliaris	6.5–6.6	–	Indirect estimation from acid production at fertilization	Rees et al. (1995)

fertilization. For this reason, the aims of the present study were to investigate the effects of external CO_2-derived pH changes on pH_i of eggs before fertilization and to elucidate if putative intracellular pH changes in relation to time of exposure would impair fertilization and lead to morphological detectable cellular alterations. We conducted fertilization experiments using seawater with different concentrations of CO_2 representing historical and present conditions and different future acidification scenarios. In addition, we measured changes in pH_i levels using BCECF/AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester derivative) in unfertilized eggs exposed for 1 h to acidified seawater. For fluorometric measurements of pH_i, we used five acidification treatments (measured values around ~180, ~380, ~750, ~1,000 and ~2,000 µatm). For all experiments, the acidification scenarios were obtained by aerating filtered seawater with different CO_2 partial pressures.

Materials and methods

Collection and maintenance of sea urchins

Adult *S. droebachiensis* were collected in June 2011 by SCUBA divers in Kongsfjordneset, Svalbard between 10–12 m depth, at water temperatures around 1–3 °C. Animals were individually transported in cooled Kautex boxes half-filled with seawater to the aquaria at the Alfred Wegener Institute Helmholtz Center for Polar and Marine Research (AWI) in Bremerhaven (Germany) within 24 h after collection. They were maintained in 250–350 l recirculation seawater aquaria connected to EHEIM Ecco Pro filters (with integrated mechanical–biological filtration units) filled with North Sea seawater at 2–3 °C in temperature controlled laboratories. The light regime was set according to their natural habitat at the time of collection (daylight is continuous between May and August) for 4–8 weeks before use. Sea urchins were fed ad libitum with algae (*Laminaria* spp. and *Fucus* spp.) or mussels (*Mytilus edulis*) maintained in aquarium facilities of the AWI, because inclusion of animal protein and carotenes in the diet of *S. droebachiensis* enhances its somatic and gonadal growth (Meidel and Scheibling 1999).

Experimental setup

Acidified seawater (ASW) was prepared using North Sea seawater (averaged salinity 30.8) filtered (FSW) through a 0.22 µm Millipore nitrocellulose membrane (Millipore, Ireland). ASW was continuously aerated with humidified filtered air (0.2 µm) of different partial pressures of CO_2 in 5-l Duran borosilicate bottles equipped with glass frits. This manipulation method is the most appropriate to reproduce the expected situation influenced by atmospheric CO_2 concentrations (Riebesell et al. 2010).

The CO_2 partial pressures were selected according to the literature (Riebesell et al. 2010; IPCC-Report 2007) to represent atmospheric conditions during the last glacial maximum and the present conditions (measured pCO_2 between 180–425 µatm), projected acidification scenarios by the end of the century and increased/extreme values (acidified conditions with measured pCO_2 ~550–800, ~1,300 and ~2,000 µatm).

A custom-made gas flow controller generated gas mixtures of 180, 380, 980 and 1,400 µatm in a mass flow controller based system (CGM 2000 MCZ Umwelttechnik, Germany). This was achieved by mixing CO_2-free air <1 ppm CO_2 (Dominick Hunter, Germany) with pure CO_2 (Air Liquide, Germany). The extreme values of CO_2 were obtained by aerating FSW with a gas mixture containing 3,000 µatm. Seawater was sparkled for at least 96 h (to ensure equilibration), and targeted pH values were controlled immediately after aeration. ASW was stored at 3 °C in 1–2-l Duran borosilicate bottles without headspace until use at the same day.

Simultaneous measurements of water parameters during experimentation were not possible. But additional water

Table 2 Chemical properties of the experimental seawater used for the fertilization experiments

Experiment	Treatment	Salinity (‰)	A_T (μmol kg^{-1} SW)	pH* (NIST)	C_T (μmol kg^{-1} SW)	pH_C (Total/NIST scale)	pCO_2 (μatm)
Fertilization	~180	31.3 ± 0.1	2,419.5 ± 0.7	8.09 ± 0.00	2,165.0 ± 5.9	8.34 ± 0.01	193 ± 7.5
Test 1	~380	31.3 ± 0.1	2,396.1 ± 2.8	7.98 ± 0.00	(§)	**8.21 ± 0.00**	**351 ± 0.6**
	~550–800	31.2 ± 0.1	2,404.5 ± 0.7	7.82 ± 0.01	2,346.5 ± 3.6	7.85 ± 0.01	683 ± 22.9
	~1,300	31.3 ± 0.1	2,408.5 ± 0.7	7.78 ± 0.03	2,406.2 ± 8.6	7.66 ± 0.04	1,089 ± 101.0
Fertilization	~180	30.5 ± 0.1	2,423.3 ± 24.0	8.09 ± 0.00	2,152.0 ± 11.1	8.38 ± 0.03	175 ± 11.3
Test 2	~380	30.7 ± 0.2	2,363.0 ± 2.8	7.9 ± 0.01	2,247.3 ± 8.1	8.03 ± 0.02	425 ± 23.9
	~550–800	30.6 ± 0.4	2,398.8 ± 3.5	7.8 ± 0.01	2,322.0 ± 3.0	7.92 ± 0.00	582 ± 1.9
	~1,300	30.6 ± 0.2	2,393.3 ± 1.4	(–)	2,419.0 ± 2.8	7.57 ± 0.01	1,354 ± 22.4
	~2,000	30.3 ± 0.0	2,429.3 ± 2.8	7.58 ± 0.01	2,504.6 ± 8.1	7.42 ± 0.02	1,968 ± 99.9
Fertilization	~180	30.3 ± 0.1	2,380.0 ± 5.7	8.1 ± 0.01	2,149.3 ± 15.9	8.31 ± 0.03	207 ± 17.9
Test 3	~380	30.1 ± 0.1	2,361.5 ± 2.1	7.93 ± 0.01	2,247.0 ± 11.1	8.04 ± 0.04	423 ± 39.9
	~550–800	30.1 ± 0.1	2,362.0 ± 1.4	7.76 ± 0.02	2,321.8 ± 2.7	7.80 ± 0.01	758 ± 11.9
	~1,300	30.6 ± 0.3	2,364.0 ± 2.8	7.74 ± 0.01	2,416.3 ± 4.9	7.48 ± 0.01	1,644 ± 37.9
	~2,000	30.6 ± 0.3	2,411.1 ± 16.3	7.59 ± 0.12	2,506.5 ± 3.6	7.36 ± 0.05	2,244 ± 244.0
Fertilization	~180	30.8 ± 0.1	2,415.6 ± 2.8	7.9 ± 0.01	2,184.5 ± 26.4	8.30 ± 0.07	218 ± 40.2
Test 4	~380	30.8 ± 0.1	2,406.1 ± 3.5	7.87 ± 0.01	2,257.5 ± 6.1	8.11 ± 0.01	352 ± 9.5
	~550–800	30.6 ± 0.1	2,396.0 ± 0.7	7.69 ± 0.00	(§)	**7.90 ± 0.00**	**769 ± 0.3**
	~1,300	30.9 ± 0.1	2,446.0 ± 2.1	7.68 ± 0.01	2,444.2 ± 23.4	7.66 ± 0.10	1,118 ± 272.0

For calculations: Input conditions 20 °C, 1 d bars pressure, P and Si: 0 μmol kg^{-1} SW. Identical output conditions except for temperature (experimental temperature: 3 °C)

Values represent mean ± SD, n = 2–3 ASW subsamples per treatment. Measured conditions represent pre-industrial (~180 μatm), present (~380 μatm) and acidified scenarios (~550–800, ~1,300 μatm or higher)

pH*: A_T integrated pH measurement. pH_C: pH calculated, in total scale except C_T values were not available or were inconsistent to the treatment used **(§)**, in which case calculations were made using A_T and pH* with NIST scale (values in bold)

subsamples were taken at the beginning and end of each experiment for total alkalinity (A_T) (with integrated pH measurements), total dissolved inorganic carbon (C_T) and salinity measurements, performed due to logistic reasons within a week of collection at room temperature. The delay in the water parameter measurements is reflected in the variability observed in the measured values (Table 2).

We used A_T, pH and C_T for calculations of pH_C (pH calculated, in total scale if not stated otherwise) and other seawater parameters at the experimental temperature (3 °C). A_T was measured with a fully automated titration system (Schott TitroLine Alpha Plus, precision of ±13 μmol kg^{-1}) and C_T with a Technicon TRAACS0 continuous flow analyser (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.

Seawater speciation and pH at experimental conditions were calculated from measured A_T, pH and C_T values with CO_2SYS software (Lewis and Wallace 1998), using dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). In cases where C_T measurements were not available, pH_C in NIST scale was calculated.

Fertilization experiments

Fertilization experiment procedures were performed according to the literature (USEPA 1993; Havenhand et al. 2008; Byrne et al. 2009; Bay et al. 1993; Kurihara and Shirayama 2004; Lera and Pellegrini 2006). For each fertilization experiment, 4–6 females and 2–4 males were induced to spawn by intracoelomic injection of 1 ml KCl 0.55 M. Females shed the eggs into filtered seawater at 2–3 °C. Sperm was collected dry and kept in 2-ml Eppendorf Safe-Lock Tubes on ice until use. We assessed the gamete quality under a light microscope before use: Sperm quality was determined by motility and fertilization capability and egg quality by shape, size, presence of visible nucleus and fertilization competence. Eggs were washed and sieved through mesh screens 40 (425-μm pore) of a Cell Dissociation Sieve-Tissue Grinder Kit (Sigma-Aldrich Co. St Louis, MO USA) using filtered seawater. This step was necessary for eliminating rest of KCl and fecal debris. Afterward, gametes from both sexes were pooled for minimizing variability due to parental effects, procedure that is of common use in fertilization assays. Selected sperm was diluted 2–3 min before the experiments in ASW to a final dilution of 10^6 sperm ml^{-1} in each experimental

vial. We selected this concentration according to Dale and Monroy (1981), Dinnel et al. (1987) and Levitan (1998b).

We let fertilization proceed for 1 and 3 h (Fertilization time, Ft). We used these fertilization periods to assess the formation of the FE and at least the first cellular divisions. We set three replicates per treatment (3 replicates × 2 fertilization time × 4–5 acidification levels per experiment). Experiments were performed in 25-ml Duran borosilicate glass Erlenmeyer filled with ASW and closed with a silicon cork. After 1 h and 3 h Ft, we concentrated the eggs/zygotes in each replicate to 9 ml volume and fixed them with 1 ml formalin 10 % in seawater for posterior analysis. We analyzed all fixed samples with 100× magnification using an Olympus BH-2 microscope (Japan) connected to an AxioCam ICc1 and linked to a computer equipped with the software AxioVision (Vers. 4.6.3.0, ZEISS). A Leica TCS SP5II multiphoton laser microscope (Leica Microsystems MS, Wetzlar, Germany) was used to investigate the position of sperms in relation to the FE in samples showing sperms in the perivitelline space (PVS) in more detail.

In sea urchin fertilization test protocols, toxicity is indicated by a reduction in the percentage of fertilized eggs (only assessed by the correct FE formation) when compared to a control sample (USEPA 1993, 2012). In our experiments, we classified the eggs by the FE formation and in addition by atypical changes in the hyaline layer (HL), the presence of cellular division (consider as successful fertilization) or by cellular abnormalities after fertilization (as a sign of unsuccessful fertilization). All cells of each replicate in our experiments were classified into two main categories: "successful fertilization (SF)" and "unsuccessful fertilization (UF)" which included subcategories (see Table 3). Eggs with no defects in the FE (PFE for perfect FE or no lesion) or small defects such as hyaline blebs (HB) and partial lifting-off of the FE (PLO) were included into SF due to the achievement of cellular division. In these cases, we gave the lowest lesion scale to those eggs showing perfect FE but with HB. The following subcategory comprised eggs with a well-formed but partially lifted FE, even when partially lifted FE may enhance the penetration of more than one sperm (Longo and Schuel 1973). Eggs classified as UF included no lifting-off of the FE (NLO), unfertilized eggs (UnF) and fertilization failure (FF). Impairment in the FE formation may be involved in polyspermy events as documented in the literature (Vacquier et al. 1973; LaFleur et al. 1998; Haley and Wessel 2004b; Levitan et al. 2007; Runnström and Manelli 1964; Longo 1978; Longo and Schuel 1973; Hiramoto 1962). For this reason, eggs in the highest lesion scale exhibited one or more pathological changes, such as irregular divisions, protrusion and bulges formation or cytoplasmic degeneration, together with abnormalities in the FE and/or HL. Eggs with numerous sperms in the PVS were included in this subcategory.

Fluorometric measurements of intracellular pH

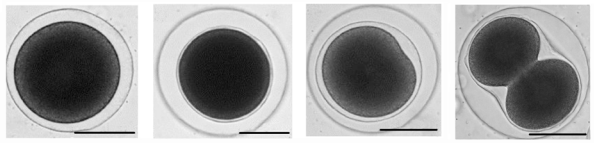
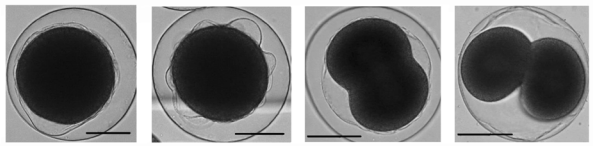
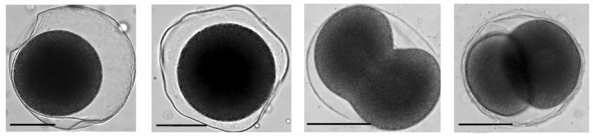
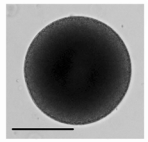
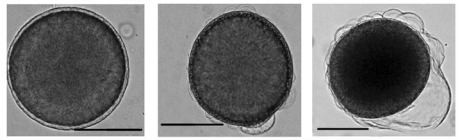
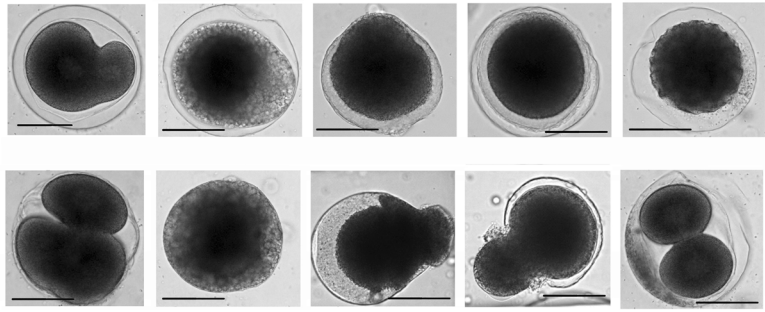
We exposed unfertilized eggs from individual females to different acidification treatments to evaluate the response of the eggs to CO_2-simulated acidification scenarios (Table 4). Pre-incubation simulates scenarios of asynchronous spawning events which are also observed in nature. In such situations, gamete contact occurs either immediately or after some time after shedding. This may derive in variable periods of exposition to acidified detrimental conditions before fertilization.

We measured pH_i changes using BCECF–AM 1 mM stock solution in DMSO (Invitrogen Molecular Probes, Oregon USA) diluted in ASW to 5 µM final concentration. Ratiometric measurements of pH were conducted using the pH excitation profile of BCECF–AM, which is pH dependent. This method has been successfully used in cell cultures (Dascalu et al. 1992; Ozkan and Mutharasan 2002), rat oocytes (Ben-Yosef et al. 1996), *Xenopus laevis* oocytes (Sasaki et al. 1992), recently in the marine platyhelminth *Macrostomum lignano* (Rivera-Ingraham et al. 2013) and sea urchin eggs of *Paracentrotus lividus* (Ciapa and Philippe 2013).

Eggs were incubated in closed 5-ml brown bottles (final volume without headspace) for 30 min on ice. Screw caps were equipped with PTFE septa. Under such conditions, no gas exchange is possible allowing us to maintain stable water parameters. The ASW within each bottle was then exchanged by new ASW and the dye BCECF–AM was added. Eggs were incubated with the dye for 30 min. Subsequently, 10 µl of egg-containing medium were taken out and placed on a measuring chamber containing 5 ml ASW of the corresponding pH at 3 °C (Table 5).

Fluorescence was obtained through an UV objective (Zeiss NeoFluor 20X) using excitation wavelength of 490 and 439 nm. Emission intensity was detected at 535 nm. Fluorescence intensities were recorded 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" tool of the software (Metafluor, Meta Imaging Series). Water subsamples for measurements of water parameters (two per treatment) were collected immediately after ASW exchange in 100-ml Duran borosilicate bottles with silicon septa under the screw cap and kept at 3 °C until measurements were performed as previously described.

Table 3 Eggs classification according to three main features: presence and state of the FE, state of the HL (when visible) and achievement of cellular division

Category	Subcategory	Description	Appearance
SF	**PFE**	**P**erfect **FE** surrounding a central egg. The HL, visible or not, is tightly in contact with the surface of the egg or slightly separated from it. Cellular division may proceed.	
	HB	Eggs within a perfectly formed FE. The **H**L **B**lebs (with different degree of separation) around the surface of the eggs, forming in most cases a "rosette-like" structure (•). Cellular division may proceed.	
	PLO	The FE is partial lifted-off at one or more sites around the egg. The HL, when visible, is attached to the egg. Cellular division may proceed.	
UF	**UnF**	Eggs which did not succeed to be fertilized: with no sign of FE formation or HL and no deformation of the egg's surface, typically observed between fertilization and cellular division.	
	NLO	Eggs with FE formation but no lifting-off. HL may be visible or not. Both structures can be seen as a halo around the egg or as blebs in a "rosette-like" structure (•).	
	FF	a. Irregular division with perfect or partially lifted FE or without it. HL with blebs may be observed. b. Protrusions/bulges (*), cytoplasm vacuolization or degeneration (detected as a gradual density change) within a perfect or partially lifted FE or without FE. c. The FE is formed around the egg but it contains sperms in the perivitelline space. Cell division may proceed.	

(•) term used by Epel (1975)/(*) term used by Runnström and Manelli (1964)

SF successful fertilization, *UF* unsuccessful fertilization, *scale bar* 100 μm. Subcategories are explained in the text

Table 4 Chemical properties of the experimental seawater used for intracellular pH measurements. Input conditions: 20 °C, 1 dbars pressure, P and Si: 0 µmol kg^{-1} SW. Identical output conditions except for temperature (3 °C)

Experiment	Treatment (ASW)	Salinity (‰)	A_T (µmol kg^{-1} SW)	pH* (NIST)	C_T (µmol kg^{-1} SW)	pH_C (total scale)	pCO_2 (µatm)
pH_i	~180	31.2 ± 0.1	2,383.8 ± 1.0	8.13 ± 0.00	2,129.1 ± 9.5	8.34 ± 0.01	192 ± 3.5
	~380	31.3 ± 0.2	2,396.2 ± 5.1	8.05 ± 1.41	2,258.1 ± 4.4	8.08 ± 0.01	379 ± 6.7
	~750	31.2 ± 0.1	2,382.2 ± 2.1	7.63 ± 0.01	2,350.1 ± 19.8	7.80 ± 0.02	770 ± 46.2
	~1,000	31.1 ± 0.1	2,398.2 ± 7.8	7.58 ± 0.03	2,385.0 ± 24.6	7.71 ± 0.10	980 ± 229.2
	~2,000	31.4 ± 0.3	2,448.0 ± 1.1	7.20 ± 0.00	2,529.1 ± 9.3	7.39 ± 0.03	2,110 ± 139.4

Values represent mean ± SD, $n = 2$ ASW samples per treatment. pH*: A_T integrated pH measurements

Table 5 Modeling analysis of pH and Ft effects on the proportion of successfully/unsuccessfully fertilized eggs of *S. droebachiensis*

Models	$\chi 2$	*df*	*p* value	Conf.Int 2.5–97.5 %
cbind (SF, UF) ~ pH + Ft + pH:Ft + (1|Exp) + (1|Nr)	**pH** (24.446)	1	7.6e−07***	(1.54)–(3.60)
	Ft (1,868)	1	0.172	(−1.10)–(6.08)
	pH:Ft (1,580)	1	0.209	(−0.74)–(0.16)
cbind (SF, UF) ~ pH + Ft + (1|Exp) + (1|Nr)	**pH** (68.427)	1	<2.2e−16***	(1.51)–(2.46)
	Ft (7,251)	1	0.007**	(0.05)–(0.35)
cbind (SF, UF) ~ pH + (1|Exp) + (1|Nr)	**pH** (63.892)	1	1.3e−15***	(1.49)–(2.48)
cbind (SF, UF) ~ Ft + (1|Exp) + (1|Nr)	Ft (4.3114)	1	0.03786*	(0.01)–(0.40)

Significance levels: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), $N = 106$

Statistical analysis

The main response variable (fertilization success) in the present study was the proportion of eggs which succeeded or failed in the fertilization process (SF/UF) per replicate. This was coded in the data in a form of a two-column matrix adding up the correspondent subcategories into SF or UF (where UF = Total Count–SF). For the statistical analysis, this variable was recorded in R by means of the function cbind: fertilization success ← cbind (SF, UF) as described in Grueber et al. (2011). The subcategories were additionally analyzed for their relation to pH. In all cases, variables were analyzed as percentage of total count to normalize for different cell numbers per observation from different experiments. Data exploration was based on Zuur et al. (2010) and included the test of normality and homoscedasticity assumptions as well as the presence of outliers for each category/subcategory. We conducted the exploratory statistical analysis and modeling using R Version 2.12.0 (R Development Core Team 2010).

The effects of acidification and time of exposure on fertilization were tested using a multivariate generalized linear mixed-effects models (GLMM) with logit-link function for proportional data from lme4 package (Bates et al. 2012). The predictors used were pH and Ft (covariates, fixed factors). 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 each assay and observation in order to account for the same origin of samples, possibly leading to different clusters of the data. We separately analyzed data from the pre-incubation experiment (pH_i). For statistical analysis of ratiometric measurements of pH_i, we used a nonparametric test (Kruskal–Wallis H Test). Post hoc analysis and comparison of acidification levels was performed with Dunn's multiple comparison test. The level of significance for all tests was $\alpha < 0.05$. This analysis was made using IGOR Pro Version 4.

Results

Analysis of OA effects on fertilization success

The average fertilization success depended on the level of acidification used but was independent from the time of exposure (Fig. 1). Fertilization success was lower at acidified levels ~2,000 µatm in all experiments.

The proportion of eggs being successfully fertilized can be explained by the different CO_2 scenarios used. pH had a clear and significant effect on the proportion of eggs classified as PFE, which decreased, and NLO, which increased significantly at measured acidification levels

~2,000 µatm. Perfect and smooth HLs in contact with the surface of the eggs were rare under highly acidic conditions. The proportion of eggs within HB decreased with increasing pCO_2 values after 1 h of exposure while after 3 h the treatments ~550–800 and ~1,300 showed no difference to pre-industrial and present conditions (Fig. 2a). This may be interpreted as a delay in the HL formation. In addition, after 3 h, zygotes in the 2-cell stage showed in some cases totally or partially dissociated cells within a distended HL (Fig. 2b). HB varied in the degree of blistering (Fig. 2c). Enlarged HLs were also observed in the PFE subcategory but at a lower frequency and only after 3 h Ft.

The proportion of eggs included in the UnF, FF and PLO subcategories seems not to be significantly related to pH changes. However, there were lower proportions of eggs showing PLO features at 3 h in relation to 1 h Ft. In addition, eggs included in the FF subcategory comprised

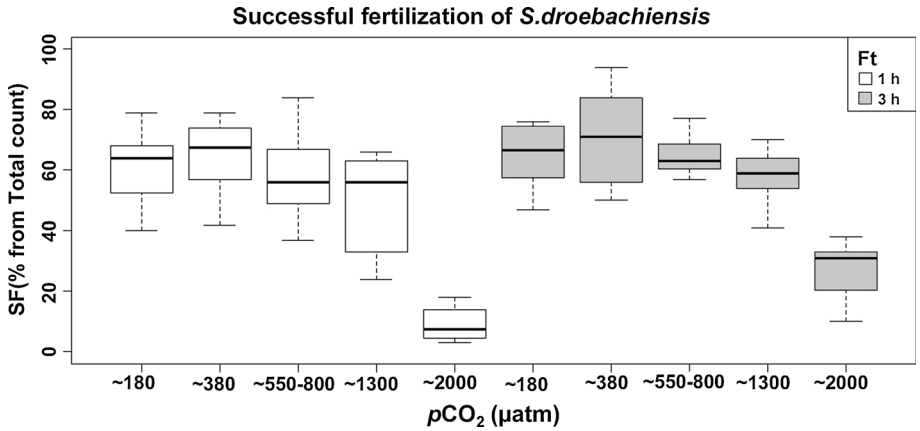


Fig. 1 Average fertilization success of *S. droebachiensis* under five CO_2-simulated acidification scenarios at two different fertilization times. pCO_2 significantly different for ~2,000 µatm treatment. Significance level: $p < 0.001$ (***), ($N = 106$)

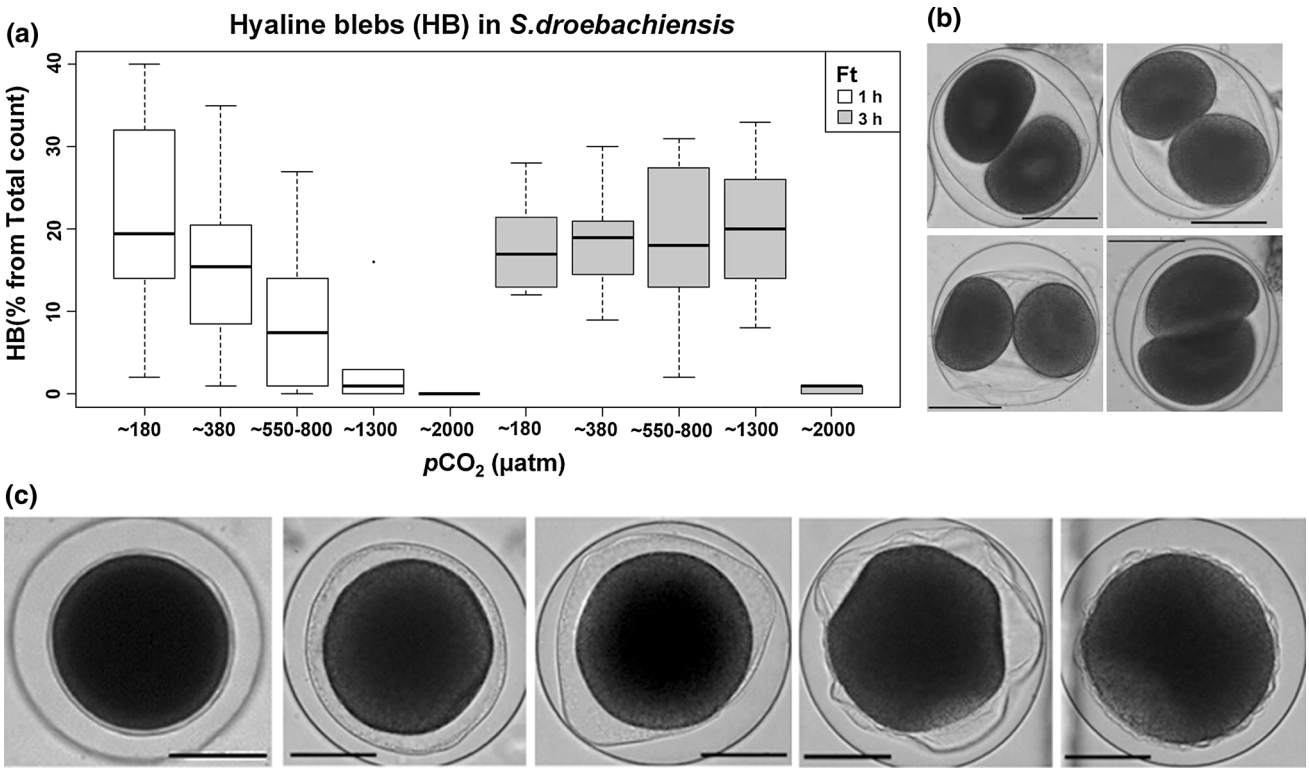


Fig. 2 **a** Proportion of eggs of *S. droebachiensis* showing hyaline blebs under five CO_2-simulated acidification scenarios ($N = 106$). **b** Eggs with inaccurate orientation after first cellular division. **c** Different hyaline layer blistering patterns. The first image represents a perfect fertilization without disorders in the HL. *Scale bar* 100 µm

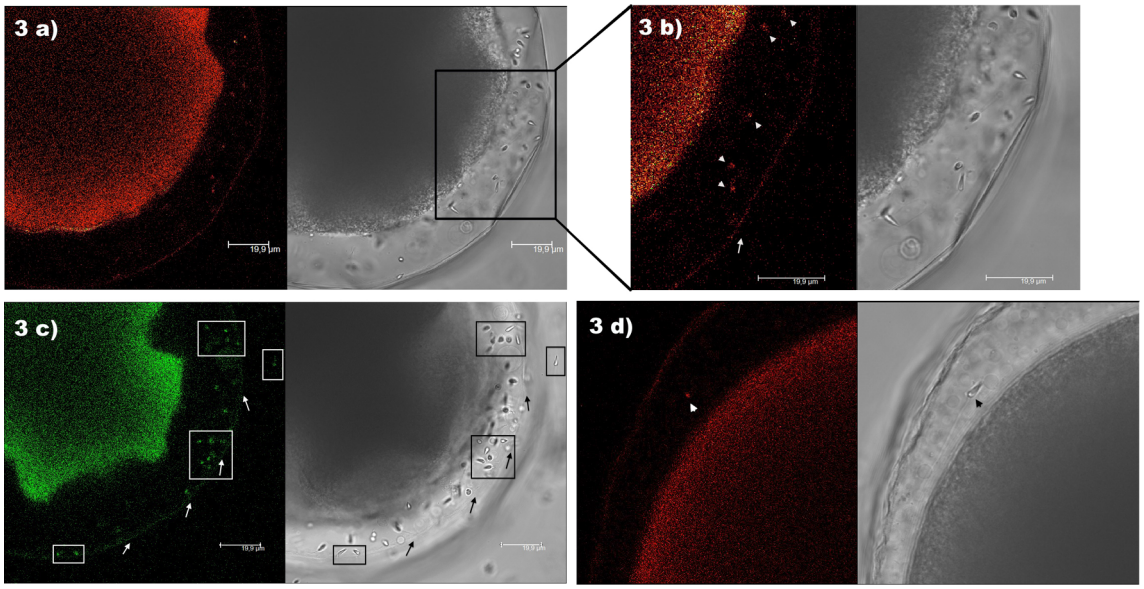


Fig. 3 **a** Sperms located in the PVS. Eggs and sperms showed auto-fluorescence in confocal images (*left dark fields*). **b** Details of indicated region in 3a. **c** Another section plane of the same egg in a. *White* and *black arrows* show the position of the FE; *white* and *black* insets indicate the sperms that can be conspicuously observed in the fluorescence image. **d** A sperm within the PVS (*arrow heads*) with a visible flagellum (*light field*)

anomalies showing no, well- or partially formed FE together with cytoplasmic disruptions and/or sperms in the PVS. These anomalies were included into one single subcategory for the presentation of results. Therefore, this subcategory must be carefully analyzed as it comprises different kind of defects that cannot be easily separated. The formation of cytoplasmic protrusions and bulges in our study is similar to those described for polyspermic events, but we could not demonstrate the entrance of more than one sperm into the eggs.

The presence of sperms in the PVS was carefully analyzed in order to assess the exact position of the sperms and to exclude the possibility of an artifact due to the treatment, the fixation procedure or the detection method. Pictures were taken at different section planes of the eggs. Due to auto-fluorescence of eggs and sperms, it was possible to observe the exact position of the sperms in relation to the FE (Fig. 3).

Intracellular pH in eggs exposed to CO_2-induced OA

The results derived from the fluorometric measurements of pH_i are expressed as the fluorescence ratio 490/439 of BCECF–AM within each measured egg. After 1 h of exposure to different acidification levels (measured values between 180–2,100 µatm), our results showed significantly different fluorescent ratios for ~1,000 µatm and higher (Fig. 4) when compared with measured 180, 380 and 750 µatm treatments ($H = 45.4$, $df = 4$, $N = 126$, $p < 0.01$ and 0.001 and Table 6).

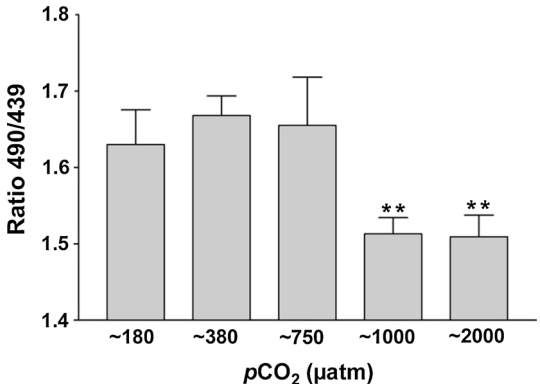


Fig. 4 Effects of the exposure to CO_2-induced OA on the intracellular pH of *S. droebachiensis* unfertilized eggs. *Error bars* represent SD and (**) significant change in pH_i ($N = 126$, $p < 0.01$)

Discussion

OA effects on fertilization success

In toxicological monitoring, successful fertilization is currently assessed through standardized protocols using sea urchin fertilization tests by the presence of a well-formed and lifted FE surrounding a fertilized egg and its HL (USEPA 1993, 2012). The restriction of the fertilization success only to this criterion may lead to possible confounding results as the morphological features of fertilization success vary in different sea urchin species. In addition, there is a long list of substances that can initiate

parthenogenesis on eggs leading to FE increase without being fertilized (Carr et al. 2006; Levitan 2004). Also, in species such as *S. franciscanus,* polyspermic eggs do not raise the FE (vitelline envelope) and do not differ from unfertilized eggs (Levitan 2004). Our morphological evaluation evidenced that criteria of fertilization success experiments should be extended to alterations of cellular structures other than the FE, as these may also point toward impaired processes.

Recent studies on later developmental stages describe how OA may result in developmental delay but also have an impact on survival rates, calcification and growth patterns of larvae from different sea urchin species (Sheppard Brennand et al. 2010; Stumpp et al. 2011a, b; Evans et al. 2013).

Our results evidenced clear detrimental effects of OA on fertilization events preceding larval formation. These findings are consistent with the reduction in fertilization rates and development efficiency referred in the literature in similar manipulation experiments for *S. franciscanus* (Reuter et al. 2011; Dupont and Thorndyke 2009), *H. pulcherrimus* and *Echinometra mathaei* (Kurihara and Shirayama 2004; Kurihara et al. 2004). Nevertheless, all these results must be carefully compared hence, under different manipulation procedures and experimental methodologies, acclimation might play an important role, as refereed by Dupont et al. (2013). In their experiments, they demonstrated that the observed decreases in fecundity and larval settlement success can be ameliorated by long-term pre-exposure of the adults with exceptions when the larvae and juvenile stages are also challenged by acidified conditions. Though, even when adults might be able to acclimate, the impact of OA will persist for the next generations. Furthermore, individuals of the same species from different populations might respond differently to acidified conditions. The results of Dupont and Thorndyke (2009) on *S. droebachiensis* confirmed a positive effect in an increased proportion of larvae reaching metamorphosis under acidified conditions, while in the present study, albeit both studies focus on different end points, acidification negatively affects the fertilization process.

Levitan (2002) stated that organisms with external fertilization are strongly influenced by population density as fertilization success varies between high- and low-density populations. Under asynchronous spawning scenarios, gametes may face similar situations like those afforded by low-density populations in which gamete contact matters. Under these circumstances, changes in pH_i may also threaten fertilization by inducing cellular alterations due to prolonged OA exposure periods. In our study, pH had significant effects on the frequency of PFE and NLO for high-acidity levels. This finding could be explained by deterioration or by an increasing inability of the eggs to cope with the measured pH_i changes, having critical consequences on the process of FE lifting-off and are additionally in line with the findings of Reuter et al. (2011). These authors revealed a reduced efficiency of the fast block to polyspermy in *S. purpuratus* under increased pCO_2 exposure and concluded that acidification might intensify the low fertilization rates typical of low-density population or of individuals inhabiting turbulent waters.

The decrease in the proportion of eggs classified as PLO and NLO from 1 to 3 h fertilization time may indicate a delay in the FE formation. A proportion of eggs within the PLO subcategory accomplished the first cell division while being attached to the partially lifted FE. Only under extreme acidification levels, PLO at 3 h was higher than at 1 h. This supports the idea of an altered FE formation even when further cellular activation mechanisms progress into an accomplished cellular division.

Under our experimental conditions (seawater enriched with CO_2), additional pathological phenomena such as HB, or vacuolization and degeneration of the cytoplasm and irregular cell division emerge. Abnormal cleavage patterns have been commonly associated to manifestations of polyspermic events in sea urchins (Levitan 2004).

Studies on hyaline focus on its role as substrate for cell adhesion during embryonic development (Wessel et al. 1998) and gastrulation processes (Citkowitz 1971). There is, to our knowledge, not much information about its role in the period between fertilization and the first division. The HL might keep the cells in the right orientation for division. Citkowitz (1971) referred that the HL is firmly

Table 6 Kruskal–Wallis H test and post hoc Dunn's multiple comparison test for fluorometric ratios per treatment

Treatment comparison test	~180 µatm				~380 µatm			~750 µatm		~1,000 µatm
	~380 µatm	~750 µatm	~1,000 µatm	~2,000 µatm	~750 µatm	~1,000 µatm	~2,000 µatm	~1,000 µatm	~2,000 µatm	~2,000 µatm
Difference in rank sum	−14.05	−4.10	38.41	37.61	9.95	52.46	51.66	42.51	41.71	−0.80
p value	ns	ns	**	**	ns	***	***	***	***	ns

Significance levels: $p < 0.01$ (**) and $p < 0.001$ (***), $N = 126$

attached to the embryo, is not lost until metamorphosis of the larvae and becomes difficult to observe when it is partially digested or weakened. This author stated also that the HL can be synthesized de novo by the embryos. Nevertheless, its inadequate formation may imply unsuccessful orientation of cells during and after cell division (as observed in the present study).

In our experiments, the proportion of eggs showing HB increased with the time course of exposure, with higher interspaces observed in higher acidified levels. It might be possible that the HLs in our experiments after longer exposure of the eggs become destabilized or degraded due to the acidic environment. This could lead to the different HL blistering patterns or to its absence. Blebs could be a precursor stage of a distended HL and could directly lead to an inaccurate cell orientation after division.

The literature approaching the effects of pH on HL and FE formation in the context of cell biology is scarce. Only few studies described the phenomenon of blebbing. Epel (1975) describes that improper increase in the FE in the presence of trypsin inhibitors rends eggs that looks like "bubbly rosettes" and he concluded that the vitelline layer (FE precursor) is attached to the plasma membrane by protease-sensitive bonds. He mentioned that within the cortical granules, there are two kinds of proteases which are released upon fertilization and that are involved in different processes: detachment of the vitelline layer from plasma membrane and detachment of supernumerary sperm from the still not hardened FE. Posterior studies identified and characterized the proteins involved in the process of cortical granules exocytosis (Haley and Wessel 2004a, b, 1999; Wong and Wessel 2008) and observed fertilized eggs with similar "blebby" envelopes, after treatment in artificial seawater adjusted to pH 8, 7, 6, 5 and 4 as in our study. 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 contributes to the block to polyspermy.

Disturbance or inhibition of the CGSP1 activity may be a possible explanation for the presence of detached FEs with numerous sperms still linked to their surface indicating impairment in the FE formation and disturbances in the spermicidal function of the proteases involved in the detachment of supernumerary sperms.

An alternative explanation for impaired FE formation and sperms: egg contact may be related to disorders in the extracellular matrix (ECM). OA may disrupt gametes interaction through elements of the ECM in the egg coat. Fucose sulfate glycoconjugates (FSG) and sperm-activating peptides (SAP) are both main components of the ECM involved in the Ca^{2+}- and pH_i-dependent activation at fertilization. Hoshino et al. (1992) studied the effects of two extracellular pH conditions (pH_o 8 and 6.6) on FSG and SAP and its consequences on the intracellular levels of Ca^{2+} (Ca_i^{2+}) and pH_i. These authors found that at pH_o 6.6, these ECM elements do not induce the expected increase in Ca_i^{2+}. Moreover, at these levels of extracellular pH, an increase in pH_i is only induced by exposure to SAP. Based on these results, even when SAP seems not to be affected by acidic conditions, FSG will putatively be impaired leading to incomplete activation of the Ca_i^{2+} response. This affects the synthesis of cyclic nucleotides necessary during the acrosome reaction impairing finally the gametes interaction.

The enormous number of sperms observed in the PVS of fertilized and even dividing eggs in our samples could evidence the deterioration and weakening of the FE or an abnormal formation leading to impairment of its primary functions. Vasilev et al. (2012) referred that remnants of the cortical granules can be extruded to the perivitelline space after fertilization in eggs exposed to ionomycin. These remnants could be confounded with sperm cells due to similar size when analyzing the samples under light microscopy. We demonstrated that in our samples, the structures observed within the PVS were indeed sperms. Moreover, there were many empty FEs with sperms still attached to their outer surface which did not succeed to be separated, suggesting failures also in the spermicidal function of the proteases contained in the cortical granules.

In the present study, we observed eggs with typical features of polyspermic events (e.g., protrusion and bulges formation grouped into the FF subcategory) as well documented by Hiramoto (1962), Runnström and Manelli (1964) and Longo and Schuel (1973). Levitan (2004) referred that sperm entering the egg become difficult to track several minutes after fertilization. Nevertheless, as we did not perform a sperm staining, we cannot guarantee that the observed abnormal cleavage patterns and other cytoplasmic alterations are linked to polyspermy events.

None of the studies that have been performed in the frame of OA using fertilization tests (Kurihara et al. 2004; Kurihara and Shirayama 2004; Byrne et al. 2009, 2010; Martin et al. 2011; Reuter et al. 2011) mentioned similar cellular alterations as shown in our study, thus making comparisons not feasible. To discard a possible species-specific response, we repeated the same fertilization procedures with a second sea urchin species (*P. miliaris*) obtaining similar effects (data not shown). Apart from the differences in species and methodologies used, the published responses to OA exposure is variable ranging from significant to non-significant or inconclusive results. This could be related to different acclimation histories and pre-adaptation due to naturally different habitat conditions

(Shen and Buck 1990). The results on compensatory responses found for *S. purpuratus* larvae from the California population (Stumpp et al. 2011a, 2011b), already exposed to seasonally increased pCO_2 and hypoxia in their habitat, are consistent with a potential adaption hypothesis. Their results indicate that the developmental delay and increased metabolic rates observed under CO_2 conditions are also related to changes in gene expression patterns as compensation mechanism. In addition, studies performed with *S. droebachiensis* larvae suffering from a reduction in gastric pH due to OA exposure evidenced compensatory feeding to face decreased digestive efficiencies (Stumpp et al. 2013). These results are based on the gametes of non-exposed adults which eggs were exposed only after first division. Whether pre-exposure of adults (Dupont et al. 2013) or gametes (present study) would lead to similar or impaired larvae responses as those observed by Stumpp et al. (2013) is an interesting matter for further research.

OA effects on pH_i of pre-incubated eggs

Finely tuned internal pH modifications are important for cell activation after fertilization. Therefore, the limited buffer capacity of a stressed cell due to external acidified conditions might influence the cellular ability to respond to activation mechanisms. Cells are able to compensate for these effects by means of energy expenses. Our results showed that down to pH values of around 7.8 (at measured partial pressures of 770 µatm), the eggs of *S. droebachiensis* are still able to compensate the effects of CO_2-derived pH_o changes. Increased concentrations of CO_2 in seawater, e.g., measured 980 and 2,100 µatm, are associated to pH_i changes which overstrain the compensatory capacities of the eggs. Even when under these conditions, cellular division and development is still possible, expensive compensation processes might compromise the energy budget for further development.

The study of Hamaguchi et al. (1997) on the topic of pH_i variations due to changes in pH_o by medium containing acetic acid and ammonium motivated this research. We found a similar response only for high acidification conditions supporting the idea of a potential compensatory mechanism as long as the acidification levels do not outreach the buffer capacity of the cells. However, the experimental regulation of pH_i presented by these authors is entirely different from CO_2-related acidification in our study. These authors found that pH_i can be experimentally regulated in a wide range of pH_o after 10–20 min exposure to a combination of weak bases and acids. Under these conditions, pH_i coincide with pH_o due to passive ion transport of ammonium and protons (H^+) at different rates.

Evans et al. (2013) suggested that intracellular compensation in the frame of OA exposure might involve transmembrane movements of H^+ and bicarbonate (HCO_3^-) via Na^+/H^+ and Cl^-/HCO_3^- exchangers at expenses of energy. These authors showed that the transcription levels of the genes related to such mechanisms did not change in *S. purpuratus* larvae under exposure to acidic conditions, explained by habitat-related pre-adaption mechanisms. Similarly, the calcifying primary mesenchyme cells (PMCs) in larvae of *S. droebachiensis* are able to compensate intracellular acidosis via a Na^+-dependent HCO_3^- transport and avoiding the loss of HCO_3^- throughout the intestinal epithelium (Holtmann et al. 2013). This suggests the presence of a bicarbonate buffer system that allows calcification even at environmentally inadequate pH values (Stumpp et al. 2012a; Holtmann et al. 2013). Yet, the plasma membrane of unfertilized eggs contains a permanent and reversible Na^+/H^+ exchanger which contributes to the regulation of pH_i and Na^+ content of the eggs independently of metabolic energy (Payan et al. 1983; Girard et al. 1982). Payan et al. (1983) stated that the increased pH_i at fertilization is maintained by an Na^+/H^+ exchanger, similar to those found in the unfertilized eggs, and an energy-dependent H^+-excreting system. Further studies on compensation mechanisms in the eggs have still to be done in order to obtain better conclusions, to link published results and to elucidate the role of these mechanisms in the frame of OA during fertilization. More research is also necessary to clarify which of the already described transporters involved in the pH_i-regulatory machinery of larvae's PMCs (Stumpp et al. 2012a) are actually playing a role in pH regulation in eggs prior to fertilization, to cellular division and to the beginning of transcription processes in the zygotes.

Summary and future perspectives

Under the CO_2-simulated OA scenarios in the present study, we demonstrate that the fertilization success of *S. droebachiensis* is strongly impacted not only due to impairments on the FE formation but also by means of alterations of the buffer capacity of the eggs and changes in their pH_i. Dubé et al. (1985) demonstrated that lowering pH_i after fertilization affects processes such as protein synthesis rate, karyokinesis and cytokinesis to different extents, mainly leading to a delay or injury of these processes. At many pivotal points, the fertilization process depends also on the polymerization and localization of actin filaments and needs further research during gamete fusion, sperm penetration cortical granules exocytosis and intracellular Ca^{2+} release (Chun and Santella 2009).

Microvilli elongation during gamete fusion is due to 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 Rebhun (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. Thus, relatively small changes in intracellular pH could act as control mechanism in regulating the assembly of actin filaments, evidenced by the different patterns of actin polymerization under different pH conditions (Begg and Rebhun 1979). Nevertheless, actin polymerization depends not only on pH rises but also on the increased Ca_i^{2+} at fertilization (Dufresne et al. 1987).

Actin and tubulin are also actively involved in the activation process of metabolism and cell 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 dynamics might be threatened by exposure to acidification. Moreover, the sperm–egg contact itself may be affected in addition by cytoskeleton alterations in the egg because impairment of actin polymerization leading to unsuccessful formation of microvilli may interfere with the process of sperm engulfment. These aspects have still to be evaluated under CO_2-simulated scenarios and offer an unexplored target for future studies.

Further research is also necessary to evaluate whether exposure of adults may influence or affect the quality of the spawned eggs. Kurihara et al. (2013) stated that in *H. pulcherrimus,* hypercapnic exposure delayed gonad maturation and spawning without having effects on the maximum number of ova spawned. In contrast, Dupont et al. (2013) stated for *S. droebachiensis* that due to long acclimation periods (16 months), the decreased fecundity observed at short exposure periods (4 months) is abolished. On the other hand, several authors have reported a decrease in adult's food intake at higher pCO_2 conditions (Kurihara et al. 2013; Siikavuopio et al. 2007; Stumpp et al. 2012b) which could compromise the gametogenesis. Consequently, acclimation/adaption and exposure time are still matters that need additional studies in order to link already published results.

OA impact might be stronger than expected when considering the percentage of eggs with cellular anomalies in our fertilization tests. Pre-adaption might help to palliate OA impact in some population, but exposed animals at stable environments, like the Arctic Ocean, could face bottleneck events. Especially for species which natural habitat does not promote pre-adaption strategies, advancing in the knowledge of the mechanisms involved in reproduction and survival must be a core issue in order to forecast OA impact at higher organization levels.

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