Author comments on "Fertilization success of an arctic sea urchin species, *Strongylocentrotus droebachiensis* (O. F. Müller, 1776) under CO$_2$-induced ocean acidification" by D. Bögner et al.

General answer

We would like to thank Prof Santella, Prof Havenhand, Prof Dupont, Prof Melzner and the two anonymous referees for the time and effort invested in the critical reading of the manuscript and the valuable comments and suggestions we incorporated into our manuscript.

We agree with many aspects, especially in relation to the length of the manuscript and the complexity of the analysis of the polyspermy data. We decided to remove the results on early development in order to shorten the manuscript. More details are included to complement the information described in the Material and Methods section. The NB (untreated) treatment performed in our study will also be removed for clarity as suggested. The manuscript will be modified according to your comments with respect to the language and use of abbreviations.

Answer to specific comments
To Prof Havenhand, about the title

> *Strongylocentrotus droebachiensis* has a boreal distribution. Yet, we were interested to reflect the specific situation of a population of this species living in the Arctic. The reproductive conditions of *S. droebachiensis* clearly differ between populations from northern and southern regions in relation to temperature and season. In all cases a pre-adaption to local conditions can be expected.
>
> For example:

1) Stephens (1972) studying a population of Cape Cod Bay found that larvae of this species develop normally at -1°C, but observed that above 10 °C there is an asynchrony in the development. At temperatures higher than 12 °C there is an irreversible arrest of cell division.
2) Wahle and Peckham (1999) reported that in the Gulf of Maine and in Canadian Maritimes (southern populations) the spawning season is between March and April at temperatures ranging from 3-7°C.
3) Oganesyan (1996) refers that in the Barents Sea spawning of *S. droebachiensis* occurs at temperatures between 0.4 to 1.8 °C.
4) Himmelman et al. (2008) report spawning of *S. droebachiensis* in July at 5-7°C, while Starr et al. (1993) mentioned that spawning in the St Lawrence Estuary takes place in June-July in relation to the phytoplankton bloom. The later authors referred a citation for the study of Kaufmann (1974) in which this species spawned also in June-July at 3-5°C in the Arctic Ocean.

> We collected mature sea urchins in Svalbard between June and August, and the temperatures at the sampling site were between 1-4°C.
>
> <u>We change the title to the following:</u>

- Fertilization success of *Strongylocentrotus droebachiensis* from an arctic population under CO$_2$-induced ocean acidification.

To Prof Havenhand, Prof Dupont, anonymous referee 4 (p27 li 6-7; p30 li17 and p31 li26) about the use of NB (unfiltered seawater) treatment as control

> In the abstract/material and methods, we explicitly wrote that we used untreated filtered seawater as control. We intended to check whether the water manipulation produced any changes in the water quality. If not, the results from these samples should be similar to the data from the 380 µatm treatment. The fertilization test is very sensitive to changes in water quality, as for instance, chemicals putatively present in plastic elements of our set-up. The NB

treatment will be removed from the present study and statements about this treatment will be deleted from the text.

To Prof Havenhand (p28 li 24 ff), about introduction update

The introduction was rewritten in general and updated with new references. For instance, the sentence: "Ocean acidification (OA) is largely dependent on increasing carbon dioxide (CO_2) emissions into the atmosphere due to anthropogenic activities." is changed to:
"Ocean acidification (OA) is largely dependent on increasing carbon dioxide (CO_2) emissions into the atmosphere, mainly due to fossil fuel combustion from which coal contributed to a 40% of the emissions and oil to 36% in 2008(Le Quere et al., 2009)…

To anonymous referee 4 (p28 li 2), about addition of reference for OA impacts on calcifying animal groups

The sentence "The concentration of CO_2 in the atmosphere has a direct effect on the carbonate system of the oceans and thus, elevated CO_2 levels modify the distribution of carbonate species (Zeebe and Wolf-Gladrow, 2001), putatively compromising the life of calcifying animal groups." was rephrased and additional references are given as:
"The concentration of CO_2 in the atmosphere has a direct effect on the carbonate system of the oceans and thus, elevated CO_2 levels modify the distribution of carbonate species (Zeebe and Wolf-Gladrow, 2001). This may compromise the life of calcifying groups as previous studies have shown (Orr et al., 2005; Li and Gao, 2012; Movilla et al., 2012; Navarro et al., 2013).

To anonymous referee 4 (p28 li 8-10), about language revision

The sentence "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." was rewritten as:
"Sea urchins and other broadcast spawning species might be particularly vulnerable to ocean acidification, especially during critical phases of their life cycle, like fertilization and post-larval development."

To anonymous referee 4 and 6 (p28 li 13-21) about selection of the most relevant references

The paragraph was rewritten as:

Studies on OA effects on different sea urchin species have already been done (Havenhand et al., 2008; Clark et al., 2009; Byrne et al., 2010; Dupont et al., 2010; Martin et al., 2011; Reuter et al., 2011; Stumpp et al., 2011; Albright et al., 2012; Foo et al., 2012; Schlegel et al., 2012; Evans et al., 2013; Gianguzza et al., 2013). These results report the responses of different sea urchins species under a variety of experimental approaches in the frame of OA comprising all life cycle stages. Species may react different, depending on their acclimation history. Reuter et al., (2011) stated that the variability in response observed in sea urchins may be attributed to the different methodologies used. This limits the comparability of results, especially in studies using different seawater manipulations /CO_2 concentrations, sperm:egg ratios and endpoints. Alternatively, the diversity in experimental routines leads 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 process of fertilization envelope (FE) formation as a crucial parameter to be taken into account. To our knowledge, there are no studies on CO_2-induced OA effects on the morphology of the eggs 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.

To anonymous referee 4 (p28 li 22-25 and 27-29) about citation stating why are sea urchins of high value in differ kind of studies.

The paragraph will be rephrased as:

Sea urchins are of high value in studies on fertilization and development on account of the amount of identical material that a single sea urchin can offer, the transparency of the larvae, and the ease of adult maintenance in captivity (Mantraga, 2005). Due to these features and the reliability of fertilization assays as screening test, sea urchins are of common use as test organisms for genomic, embryology and toxicology research and also in ocean acidification research (Epel, 1998; Wessel et al., 2000; Wong and Wessel, 2005; Epel et al., 2006; Dupont et al., 2010).

To Prof Dupont, Prof Melzner and anonymous referee 4 (p29 li 4) about update of citations on OA effect studies using *S. droebachiensis*

The sentence was rewritten as:

Moreover, *Strongylocentrotus droebachiensis* have already been used in other OA studies (Siikavuopio et al., 2007; Spicer et al., 2011; Dupont et al., 2012; Dupont and Thorndyke, 2012; Stumpp et al., 2012; Dorey et al., 2013), yet information from Arctic populations is scarce and to our knowledge no data exists on fertilization success under elevated pCO_2 conditions for this species.

To anonymous referee 4 (p29 li 6-7) about vulnerability of the study region

The paragraph was rewritten as:

The study of Arctic populations with a climatically restricted distribution, 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 and which stability may be threatened by other factors like increasing temperatures. *S. droebachiensis* have 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).

To anonymous referee 4 and 6 (p29 li 10-12 and 15-21) about need of rephrase to put in context the chosen population in this study within what it is known about the species in general and offer citation of environmental parameters of the study area.

The paragraph was rewritten as:

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 (Scheibling and Hatcher, 2001; Addison and Hart, 2004; Dupont et al., 2010). This species is widely distributed in northern circumpolar waters (Pacific and Atlantic coasts of North America and Arctic Ocean) (Levitan, 1998a; Scheibling and Hatcher, 2001) within a wide range of physically different habitat conditions in terms of temperature and depth. In populations of the Northwest Atlantic, spawning is triggered by phytoplankton blooms (Scheibling and Hatcher, 2001).

Cyclic environmental changes might influence the acclimation history of animal species within different populations extending their salinity and pH tolerance. The population chosen for our study inhabits shallow waters near Ny-Ålesund and can be found between 10 m (own collection data) and 200 m depth (Laudien and Orchard, 2012). This population is adapted to extreme winter temperatures and high salinity variations during summer. In Kongsfjorden, Svalbard, the seasonal melting cycles produce changes in transparency of water masses and salinity (Svendsen et al., 2002) and the spring bloom is the only predictable phytoplankton production peak (Hop et al., 2002).

To Prof Havenhand and anonymous referee 4 (p29 li 21-28) about gamete release and language corrections

The idea was rewritten as:

Meidel and Yund (2001) reported for *S. droebachiensis* an elevated fertilization level maintained over 48 h in field experiments under natural sperm release events. These authors also found that under laboratory conditions, viability of the eggs could last even 2–3 days. Therefore, we anticipate that spawned eggs in the field situation are fertile and may be exposed to lowered pH conditions before fertilization takes place for longer periods than experimental assays have shown until now.

To anonymous referee 6 (p30 li 1) about the need of a background on the change of pH_i during fertilization

pH_i measured with BCECF in unfertilized eggs of *Lytechinus pictus* range from 6.84-7.22. Upon fertilization the pH_i increased to 7.3-7.58 in this species (Shen and Buck, 1990; Rees et al., 1995). Payan et al. (1983) reported for *Paracentrotus lividus* a pH of 7.38 for unfertilized eggs and of 7.64 in fertilized ones. Johnson and Epel (1981) using DMO, referred values of 7.08 for unfertilized and 7.47 for fertilized eggs of *Strongylocentrotus purpuratus*. Hamaguchi et al. (1997) measured also the pH by microfluorometry in unfertilized and fertilized eggs in two species using pyranine as pH indicator. These authors referred for *Schaphechinus mirablis* and *Hemicentrotus pulcherrimus* 6.8 and 6.81 in unfertilized eggs, while in fertilized eggs the pH was of 7.34and 7.32 respectively. To our knowledge no data have yet been published reporting pH_i in *S. droebachiensis* eggs. This information will be added as a table in the manuscript.

To Prof Havenhand (p30 li 7) about hypothesis on changes on pH_i due to OA exposure

The sentence "Hence, we can hypothesize that there will be internal pH changes within eggs exposed to OA.": should be changed into:
"Hence, it would be of interest to know if eggs exposed to OA, react either by lowering their pH_i or are able to regulate and maintain their original internal pH values".

To anonymous referee 4 (p30 li 18) about description of the BCECF/AM abbreviation

The phrase will be rewritten as:
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 to acidified seawater for 1 h.

To Prof Havenhand, Prof Dupont, anonymous referee 4, 6 (p 31 li 1-14) about detailed description of collection and maintenance of used organisms

The text in Materials and Methods should be rewritten as:

"Adult *S. droebachiensis* were collected in June 2011 by SCUBA divers in Kongsfjordneset, Svalbard. Sea urchins were collected between 10-12 m depth, at water temperatures around 1-2°C. Animals were individually transported in cooled Kautex boxes half-filled with seawater to the aquaria at the Alfred Wegener Institute for Polar and Marine Research (AWI) in Bremerhaven within 24 h after collection. They were maintained in 250–350 L recirculation seawater aquaria at 2–3 °C temperature controlled laboratories with light regime like their natural habitat at the time of collection (daylight is continuous between May and August) for 4–8 weeks before use. Each tank was connected to individual EHEIM Ecco Pro filters (with integrated mechanical-biological filtration units). 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)".

In detail:

Sea urchins were collected in waters around the station in Ny-Ålesund, Svalbard (town center position: 78°55'24''N, 11°55'15''E). For this place MacLachlan et al. (2007) refers salinity values between 30.95 and 33.97 and Laudien and Orchard (2012) between 23 and 34.8. This species is an osmoconformer and its salinity tolerance varies among populations depending on acclimation history (detailed effects of salinity for this species have been published by Denoux 1976, Lange, 1974, Stickle and Ahokas 1974). The mean salinity in our study was 30.8 ± 0.5 which is in the range of the collection site at the time of spawning.

Sea urchins (200 individuals) were shipped to AWI at end of June 2011 and experiments were conducted between July and beginning of September 2011. The pH (NIST Scale) of the water used for maintenance was 7.963±0.05 (NIST Scale, n=32). We had access to the same water reservoir of the polar seawater circulation system at the AWI, as other laboratories. Chemical water analysis (oxygen, ammonium, nitrite and nitrate) are performed 2-3 times per week. From the same seawater, we took samples for filtering and conduction of our experiments. A_T (with integrated pH measurements), salinity and C_T values were measured on filtered seawater samples.

To anonymous referee 4 and 6 (p31 li 21) about the 3000 μatm set-up

Seawater of the 3000 μatm treatment was bubbled at 4.9°C, but cooled to the experimental temperature (3°C) in closed bottles (screw cap with silicon septa and no head space) before use. Experiments including the 3000 μatm treatment were performed simultaneously with all other treatments (180-1400 μatm). The possible influence of bubbling at a higher temperature would be that the amount of CO_2 dissolved in the water could be less than expected for 3000 μatm at 3°C. Nevertheless, this concentration was used as an extreme value. The slight decrease that might result on this procedure could be neglected, though a clear biological effect was detected.

To Prof Havenhand anonymous referee 4 and 6(p32 li 5-17) about measurements and calculations of water parameters

The paragraph was changed into: "We measured total alkalinity, A_T (with integrated pH measurements) and C_T in water subsamples taken at the start and end of each experiment. We used A_T and C_T for the calculations of the pH at 3°C (used in statistics)."

The paragraph starting with "pCO$_2$ values of control seawater…" was rewritten as:

"Seawater speciation and pH (total scale) at experimental conditions were calculated from measured A_T/C_T values with CO2SYS software (Lewis and Wallace, 1998), using dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987)."

For more details:

We measured also A_T and C_T from samples collected immediately after bubbling, which were used as references for measured values after each experiment. Salinity and pH were measured from the same water samples used for A_T and C_T measurements. The bubbled seawater was stored in an incubator at 3°C for 1h (period of gamete collection) to guaranty that all samples had the same temperature before use (though 3000 µatm treatment was prepared at 4.9°C).

To anonymous referee 4 and 6 (p32 li 19-27) about details of the amount of individuals used per experiment and the assessment of gamete quality

In this manuscript we present the results of 5 experiments (4 WOPI-without pre-incubation and a pilot study with WIPI approach –with pre-incubation). In the WOPI experiments, 4-5 females and 3-4 males were used. In the WIPI experiment, 6 females and 4 males were used. We pooled the gametes of the individuals for each experiment. We had 3 replicates per treatment (5-6CO$_2$x2Ftx3Rep: 30-36 vials in WOPI or 6CO$_2$x2Ftx3PItx3Rep: 108 vials in WIPI). For statistical analysis we took out the outliers due to mistakes in inoculation and the observation with altered water parameters. ASW means acidified seawater. Abbreviation use will be corrected in the manuscript.

The sentence "Sperm quality was assessed by motility and fertilization capability before use and egg quality by shape, size, presence of visible nucleus and fertilization ability." was rephrased as:

"We assessed the gamete quality under a microscope before use: sperm quality by motility and fertilization capability and egg quality by shape, size, presence of visible nucleus and fertilization ability."

To Prof Havenhand, Prof Dupont, Prof Melzner and anonymous referee 4 (p33 li 1) about the used sperm concentration

The exact amount of sperm concentration of dry sperm used in each experiment is presented in the Table below. In all experiments the concentration of the dry sperm was in the order of 10^{10}, and with a dilution factor to 10^4, the final concentration per vial was finally of 10^6 sperm/ml.

We selected this concentration according to Dale and Monroy (1981), Levitan (1998b) and Dinnel et al (1987). Dale and Monroy (1981) analysed the factors involved in the prevention of polyspermy and suggested a density of 10^6 sperm/ml for sea urchins fertilization under natural conditions. Levitan (1998b) refers information about fertilization success in relation with sperm to egg contact time and Dinnel et al (1987) about the sperm cell bioassays. In a second series of experiments using the pre-incubation approach, we analysed the effect of varying sperm concentrations in detail and these results are in preparation for publication.

We used different sperm to egg ratios: with similar concentration of sperms in all experiments, we varied the amount of eggs. In order to avoid changes in the water quality, we did not wash the sperm. Gametes were in contact for 1 or 3 h (Ft). Under normal conditions this procedure should not have effects on the proportion of polyspermic eggs after the successful formation of the FE. On the other hand, exposure to acidified conditions for these periods (1h and 3h) may have had effects on the stability of the FE, though we observed divided zygotes with sperm in the perivitelline space.

Experiment	Experiment Type	Mean Dry Sperm Concentration	Egg Concentration
1	WOPI	2.5×10^{10}	300
2	WOPI	2.1×10^{10}	900
3	WOPI	2.9×10^{10}	1000
4	WOPI	2.6×10^{10}	4000
5	WIPI	2.3×10^{10}	3000

The sentence in the manuscript was rewritten as:

"Sperm was diluted in the corresponding ASW 2–3 min before the experiments to a final concentration of 10^6 sperm/ml. We selected this concentration according to Dale and Monroy (1981), Dinnel et al (1987) and Levitan (1998b)."

To anonymous referee 6 (p33 li 5) about the selection of the fertilization time

We used these fertilization times (1h and 3h) in order to include the first cell division cycle. The FE may be seen in overripe eggs, thus fertilization success might be overestimated. In pilot assays performed under normal conditions (pH 8.02) we observed that after 3 h some eggs had accomplished the first division. After these periods the eggs were fixed as described in the manuscript. As NB treatment will be eliminated, the paragraph should be rephrased as: "We let fertilization proceed for 1 h and 3 h (Fertilization time, Ft) with or without previous incubation of the eggs in ASW. We used these fertilization periods to assess the formation of fertilization envelope (FE) and at least the first cell divisions."

To Prof Havenhand, anonymous referee 4 and 6 (p33 li 12-17) about the use of experimental vials with different volume and changes on tables and figures

You are completely right that we unnecessarily confused the reader by the use of differently sized containers. This choice had practical reasons and the effect of container was tested before. The results presented for the WIPI approach correspond to a pilot experiment. This approach was later on used in our experiments which results are being prepared for publication.

Our results show that the main response to exposure was similar (decrease of fertilization success at acidic conditions) independently of the use of the WOPI or WIPI approach, which demonstrates that the container size has no influence on the results. The experiment WIPI was separately analysed with a statistical model including the effect of PIt and its possible interaction with Ft.

Details on gamete concentrations and number of sea urchins used per experiment have been mentioned above. The WIPI experiment was independent from WOPI experiments; therefore, PIt 0 was also included as the control level for pre-incubation effect at each pCO_2 used.

The effects described in Table 2 and shown in the Fig 1 and 2 were observed in all experiments, independently of the approach used. This table will be modified in order to describe more clearly each observed effect and ease the nomenclature used. Figures will be modified according to the referee's suggestions and Fig 5 belongs to the experiments on early development, will be removed from the manuscript.

To Prof Havenhand, Prof Dupont, and anonymous referee 4 and 6 (p33 li 10-15) about concentration of *Rhodomonas salinas* used

The experiment on early development was conducted independently from WOPI and WIPI experiments. The maximum concentration of the *Rhodomonas salinas* was of 10^6 cells/ml. We added 50µl of this solution to 130ml vials, resulting in a cell density of 385 cells/ml. During the experimental period at 3°C and dimmed light, no further cell divisions of *Rhodomonas salinas* took place (pers. commun. U.Tillmann, AWI). Effects on larval development will be published in more depth separately in order to reduce the length of the manuscript as suggested by several referees.

To Prof Melzner anonymous referee 4 and 6 (p34 li17 and p38 li 12-21) about details on the performance of pH_i measurements and the lack of calibration data.

The fluorometric measurements were conducted as follows:

Eggs from individual females were incubated independently in closed brown bottles (final volume 5ml without headspace and with PTFE septa within the screw cap) for 30 min on ice (under these conditions no exchange is possible and water parameters remain stable). We added the eggs, washed three times with the desired ASW to eliminate the remnants of FSW from gamete collection. Then, bottles were completely filled and closed for 30 min. After 30min incubation, the ASW within each bottle was exchanged by new ASW and BCECF-AM was added.

Eggs were incubated in the BCECF solution for 30min. Then10 µl of eggs were taken out with a pipette and added to the measure chamber containing the correspondent ASW for the measurements. This step allowed us to wash out the rest of the dye and to set the measurement temperature as low as possible in the measurement chamber.

Measurements are very fast, (around 100 milliseconds per measurements), and we conducted at least five measurements per mounted sample with "the region of interest tool" of the software, which were statistically analysed as the same sample. This procedure was repeated with all the females (5 in total) and all the pCO_2 treatments (180-3000 µatm). We use reference bottles for measurements of the water parameters.

Perfusion of the eggs with ASW was not necessary and not practicable when using bubbled seawater as the perfusion solution. Perfusion causes the movements of the eggs leading to mistakes in measurements (eggs get out of focus). Moreover, during perfusion the water parameters may not remain stable due to the loss of CO_2 partial pressure: the measurement chamber is an open system and has a small volume. According to the big size of the eggs and the short measurement interval, the internal pH should not have changed when the partial pressure of CO_2 decreased in the open measurement chamber.

We planned to do the calibration after the measurements. After conducting the intracellular pH measurements, the lamp melted down and we were not able to calibrate. We conducted further calibrations with a new lamp where ratios were slightly higher than the ratios

measured in the presented study. For this reason, these results cannot be directly linked but pH values can be estimated.

For calibrations, the eggs stained with BCECF-AM as described before were also exposed to Nigericin for 20 min. Eggs were perfused for 5 min with calibration buffer, pH 8, 7.8, 7.5, 7, 6.5, and 6, cooled on ice. Then perfusion was stopped allowing eggs to settle down and to be measured as described before. In this case, as the calibration solution was buffered with HEPES-Na, the solution should maintain its pH value stable as long as the temperature is kept constant, what we achieved by cooling the measurement surface.

BCECF has been successfully used in intracellular pH measurements in cell line cultures (Dascalu et al., 1992; Ozkan and Mutharasan, 2002), rat oocytes (Ben-Yosef et al., 1996), Xenopus oocytes (Sasaki et al., 1992), and recently in the sea urchin eggs from *P. lividus* (Ciapa and Philippe, 2013).

To Prof Havenhand, anonymous referee 4 and 6 (p35 li 6-21) about the statistical analysis of fertilization experiments and ratiometric measurements of pH$_i$

We used a GLMM because our data are expressed as proportions from total count, with random and fixed factors as predictors. GLMM with binomial distribution is adequate to analyse data where the response is a proportion. As response variables, the grouping variables "successful fertilization" and "unsuccessful fertilization" were used. This was necessary due to the variety of observed effects.

Our predictors were pH (covariate, random factor), Ft and PIt (fixed factors). Salinity as a predictor was also analysed. We suspected that a component in the observed overdispersion would be related with the differences between experiments (for instance, sample origin and water batch is different between experiments). For correction of this effect, we added a random factor coding for each assay (ExpID).

The text in the manuscript should be rewritten as:

"We applied multivariate generalized linear mixed effects models (GLMM) with logit link for proportional data from lme4 package (Bates, 2012) because our response variables were count data expressed as percentage from total count. We used as response variables in the model the grouping variables successful fertilization (SF) and unsuccessful fertilization (UF). Our predictors were pH (covariate, random factor) and Ft, PIt (fixed factors). When 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 assays in order to account for the same origin of samples."

We conducted a data exploration based on Zuur et al. (2010) in which we analysed the presence of outliers (using dotplots of individual variables), normality of the data (evaluated using QQ-Plot of all response variables) and homogeneity (assessed through conditional boxplots). The main groups used for statistical model (SF and UF) were made based on the results of multi-panel scatterplot and correspondent correlation between response variables (classification will be presented in supplementary materials) as well as the presence of cellular division in each category.

In fluorometric measurements of intracellular pH the Ratio 490/439 is calculated and it changes proportionally to the external pH between values of 6.4 and 7.8. Ratiometric measurements are the approach of choice to be independent of dye concentrations. In our measurements, the ratio values had a Gaussian distribution (see below).

For exact pH$_i$ values, we conducted a calibration using Nigericin, but as we used a new Xenon lamp values are not directly comparable. The statistical analysis conducted with ANOVA is based on the Gaussian distribution of our data. We repeated the statistical analysis of the data using a non-parametric test (Kruskal Wallis H Test) and post-hoc analysis with Dunn's Multiple Comparison Test with the following results:

| Kruskal-Wallis test	
P value	P<0.0001
Exact or approximate P value?	Gaussian Approximation
P value summary	***
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	5
Kruskal-Wallis statistic	45.4

Dunn's Multiple Comparison Test

Difference in rank	Sum	P value	Summary
180 µatm vs 380 µatm	-14.05	P > 0.05	ns
180 µatm vs 980 µatm	-4.096	P > 0.05	ns
180 µatm vs 1400 µatm	38.41	P < 0.01	**
180 µatm vs 3000 µatm	37.61	P < 0.01	**
380 µatm vs 980 µatm	9.95	P > 0.05	ns
380 µatm vs 1400 µatm	52.46	P < 0.001	***
380 µatm vs 3000 µatm	51.66	P < 0.001	***
980 µatm vs 1400 µatm	42.51	P < 0.001	***
980 µatm vs 3000 µatm	41.71	P < 0.001	***
1400 µatm vs 3000 µatm	-0.8017	P > 0.05	ns

The calibration curves obtained later on with a new Xenon lamp shows that the measurements indeed worked and are realistic even when they are not comparable.

To Prof Havenhand and anonymous referee 4 (p 35 li 24-25) about the changes made on Table 1

As supplementary material, we include a table with values of water parameters per experiment. The first two sentences of the Results section will be removed from the manuscript.

To Prof Havenhand (p 36 li 13) about analysis of independence of covariates used in the model
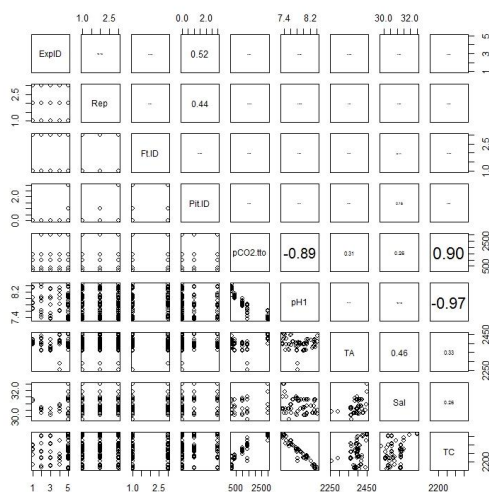
In our data exploration we analysed the relations between predictors by mean of a correlation matrix, in order to detect independence and colliniarity between the covariates/factors. The predictors analysis showed only a strong correlation between pH/pCO$_2$ and C_T, which was expected. We used pH in the statistical model and pCO$_2$ treatments were used in representation of the data and posthoc analysis of the results of the model. The rest of the predictors showed no significant interactions.

To Prof Havenhand and anonymous referee 4 (p 36 li 14-15) about the use of pH instead of pCO$_2$ in the model

Measurements of A_T and C_T are made at room temperature, therefore we calculated pH values for the temperature at which the experiments were performed. As pH values were calculated from A_T and C_T values, these data were not suitable for representation of effects. To use pH in the graphics would imply the use of a new variable dividing the calculated values into categories. For this reason, we used the treatments (pCO$_2$) for representation and pH as covariate for the statistical model.

To anonymous referee 4 about salinity (p 36 li 17-21)

The suggestion about how to handle the slightly increased salinity in 3000 µatm treatment will be added in the discussion section as follows:
The higher flow used in 3000 µatm treatment might have had influenced the salinity values of this treatment.

To Prof Havenhand, anonymous referee 4 and 6 (p 36 li 3 and 22) about the assessment of polyspermy

In table 2 we summarized the morphological observations ranked from healthy to severely damaged eggs. Changes suggested for line 22 will be incorporate accordingly into the manuscript as well as in the presentation of other results.

We agree with you in the difficulty to assess polyspermy reliably and in the risk of underestimation/overestimation by the classification of the eggs based on morphological criteria. Therefore, we rephrase our terminology in the manuscript into "risk of polyspermy".
We did not stain sperms which had entered the eggs as Franke et al (2002) did, but in our classification the morphologies classified as polyspermy are in line with those published in previous studies concerning this subject (Hiramoto, 1962; Runnström and Manelli, 1964; Longo and Schuel, 1973).

The definition of polyspermy as the penetration of more than one sperm into the egg should be carefully used as outlined below. In Gilbert (2010) polyspermy is defined as the "entrance of multiple sperm" into the egg, and more important, taking part in the genetic material exchange "resulting in a triploid zygote". Franke et al. (and most authors of the literature referenced for polyspermy) defines polyspermy as the presence of at least more than one sperm in the cytoplasm, in the case of Franke et al, evidenced by a nuclear staining. Nevertheless, this does not necessarily hints at an evidence for the participation of the second sperm in the genetic material exchange. In his study on effects of injection of spermatozoa into the egg, Hiramoto (1962) showed that the entrance of a second sperm (in his study by injection) rendered "in many cases" zygotes that divided regularly "in spite of the presence of live (sometimes moving) spermatozoa in their protoplasm". This is possible as long as the second sperm does not participate in the mitotic process. This author offers also graphic representations of "polyspermic mitotic figures" and pictures of polyspermic eggs, similar to what we observed in our study.

In the present study the main response in the experiments was independent of the approach used and we observed that the primary function of the fertilization membrane to block entry of more than one sperm failed due to OA. The presence of supernumerary sperm in the perivitelline space, especially numerous in acidic treatments, is a clear evidence for this interpretation. Nevertheless, Hiramoto (1962) showed also that the injection of sperms in the periviteline space not always leads to polyspermy, although it might increase the risk for

polyspermy to occur. There must be a time window, in which the effect of a second sperm leads to polyspermy and consequent death, and after which it is not possible for an extra sperm to take part in the mitotic process.

In cases of partial lifting-off of the FE we anticipate on the basis of the results of Longo and Schuel (1973) that there is an increasing probability of additional sperm entry into the cytoplasm imposing an additional risk for polyspermy. As in this category we observed eggs having accomplished the first cellular division, this category was included in the successful fertilization group. The degree of overestimation in our results would rely on this category. Yet, as described above, it might depend on the feasibility to form a functional FE after the first sperm entry, and before others are able to penetrate and disturb posterior division events.

As our main target was the study of the FE formation, we distinguished between polyspermic cells **with** or **without** FE in the classification. Grouping all the observations into only one category, e.g. total polyspermy, implies that the differences observed between NFE (no FE formation) and WFE (with FE formation), would be omitted. Our results show that the incomplete formation or the posterior weakening of FE plays a role in the increased polyspermic risk. The higher variability observed in the eggs classified as NFE in WIPI experiments might be an indicator of deterioration of egg integrity due to OA exposure.

We also detected eggs with irregular division, with protrusions of the cytoplasm or with phenomena like vacuolic degeneration of the cytoplasm (for detailed description see changes in Table 2). These alterations can be either due to real polyspermy as defined in Gilbert (2010) or being related to degenerative process after fertilization failure. The causes might be direct interference with the actin polymerization due to acidic conditions (Begg and Rebuhn, 1972) or impairments by other unknown mechanisms involved and not studied in the present research.

Fading and vacuolization of the cytoplasm have been reported for oocytes undergoing atresia after exposure to environmental pollutants (Schäfer and Köhler, 2009) and in autophagy during vertebrates development. Fink and Cookson (2005) refer as an endpoint for oncosis the loss of membrane integrity and stated that this process might be the result of altered intracellular Ca^{2+} levels. Oncosis is a pre-lethal pathway leading to cell death (as a counterpoint to apoptosis) which is accompanied by cellular swelling, blebbing and increased membrane permeability. These authors stated that during apoptosis the loss of cell shape might be the result of cytoskeleton proteins cleavage, and the plasma membrane blebbing may result from caspase-activation of actin depolymerizing enzymes as also reported by Voronina and Wessel (2001) in sea urchin oocytes, eggs and embryos.

We did not analyse if the cells categorized as unsuccessfully fertilized were indeed suffering from this processes, but the morphological features observed (e.g. vacuolization, swelling, and loss of cell shape) strongly point out to similar processes. We differentiated the kind of blebbing observed in our study into those in the HL and those special cases in the plasma membrane, mostly observed in acidic conditions and not included in the analysis. In any case, a deeper research about possible effects of OA on the apoptotic machinery in eggs and embryos is needed to elucidate the mechanisms explaining these observed morphologies. Changes suggested for line 22 respect to formulation of results: all suggestions will be included in the text and the results part will be modified accordingly.

To Prof Santella about Table 2

As we did not stain the sperm for detection of polyspermy and based our classification only in the defective morphology observed at cleavage, we agree with you that there is a lack of

connection between the monospermic/polyspermic activation and the lifting-off of the FE. Our classification is in line with the results referred by Hiramoto, (1962), Runnström and Manelli, (1964) and Longo and Schuel, (1973), which were very informative as these authors described in detail the morphology of what they classified as polyspermic eggs. They offer also explicative figures and pictures, which are very similar to what we observed in our results, like the formation of protrusion and bulges (in our paper ascribed as cytoplasmic constrictions-extrusion)

To properly describe our findings, we also considered the terms viable/non-viable fertilization, which were not used because of the following reasons:

1) It is described in the literature that some eggs, being fertilized by more than one sperm (though only one takes part in the mitotic division), are also able to derivate into larvae which develop with an injured endomesoderm-mesenchyme (Runnström and Manelli, 1964).

2) Within our classification of successful fertilization there are eggs with a perfectly formed FE and atypical formation of the hyaline layer (blebbing at different degree). We observed other kind of blebbing concerning the plasma membrane, probably related to apoptotic mechanisms which were not included in the statistics.

3) Our endpoint was set to a maximum of 3h after fertilization, the period in which we previously observed the first cellular divisions to occur. After this time, exposed / affected zygotes might not necessarily accomplish further successful divisions leading to healthy larvae.

4) In *Strongylocentrotus franciscanus*, polyspermy may cause the impairment in the lifting-off of the FE and eggs are similar in appearance to unfertilized eggs (Reuter et al., 2011). In line with this, even when this phenomenon it is not described for *S. droebachiensis*, we included the unfertilized eggs within the unsuccessful fertilization variable.

Independently of the classification, our data indicate that there is an effect of OA exposure on at least two steps in the fertilization process: in the FE formation and in the division.

In addition we conducted similar experiments with eggs of *Psammechinus miliaris*, that accomplish the first division after 35-40 min and needs 80 min for the 4-cell stage. In this species, we observed eggs with incomplete divisions until the 4-cell stages after 3 h from inoculation with sperm (without having completed the furrow formation necessary for cell division in both division cycles). This indicates that there might be also problems in the coupling of actin with other proteins involved in cytokinesis, especially those in charge of the furrow formation.
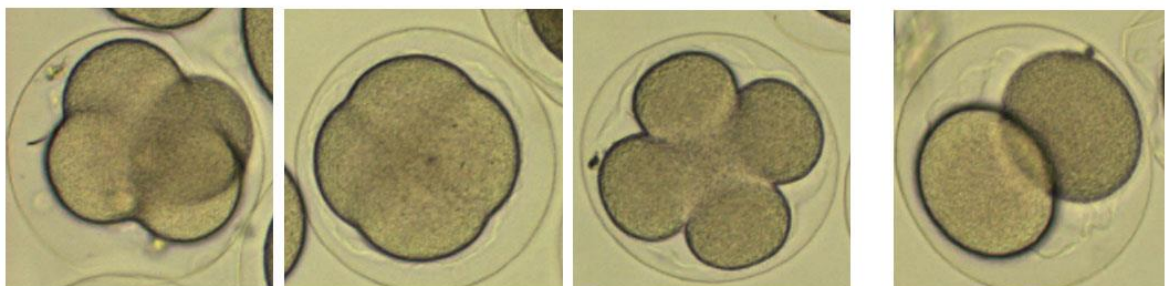
Irregular divisions until the 4-cell stage.   Perfect division with hyaline blebbing (HB)

To Prof Santella about Fig. 2

In eggs in which the FE had signs of defective formation (e.g. partial lifting-off or curly appearance of the FE), it is expected that supernumerary sperms can enter into the periviteline space. Longo and Schuel (1973) stated in their results that sperms can enter through the sites in which the FE failed to separate.

Notwithstanding the primary function of the FE is to protect the egg from multiple sperm entry and should be robust enough to protect the fertilized egg until hatching, we were astounded to find eggs with sperms in the perivitelline space. In these eggs the FE was apparently perfectly formed and zygotes were even in the 2-cell stage. This can only be attributed to a weakening of the FE structure at contact with acidic conditions or to failures in the formation process under acidic conditions. We also observed a high number of empty FE in our samples. Some of these empty FE presented attached sperms (which may be a sign of failure in the protease activity).

Wong and Wessel (2008) reported the steps involved in the formation of the FE. This process depends on many enzymatic reactions and must have a precise spatio-temporal regulation. Under the premise that in our experiments the intracellular pH changed by the treatment in different acidic conditions, it might be possible that the regulation of the enzyme involved in the lifting-off could be affected.

Cortical granules (CG) have a pH around 5. The change to alkaline conditions around pH 8 upon fertilization induces the auto-activation of the CG enzymes. If there is an imbalance in the magnitude of this change or it does not occur at the right moment and place, the risk of deregulation on the FE formation steps may increase.

We detected changes in intracellular pH of exposed eggs, primarily on acidic treatments, after pre-incubation of the eggs for 1h. In the pre-incubation experiment, we found a decrease in the proportion of eggs that achieved the FE formation successfully and an increase in the number of malformed FE in the most acidic treatments.

As we did not stain the sperms, we cannot assess the number of sperms entering the eggs, but the probability, accounting for the proportion of eggs with sperm in the perivitelline space, might have been elevated under the experimental conditions. Nevertheless, this does not necessarily mean that a successful gamete interaction occurs between the egg and the sperms in the perivitelline space. Hiramoto (1962) indicated that: "the activation of egg protoplasm from outside is necessary for the occurrence of the second phase of fertilization phenomena". Thus, in our analysis, it might be possible that we underestimated the risk of polyspermy, especially by the inclusion of the PLO group within the successful fertilization variable.

As described before, another possible explanation may be the activation of apoptotic machinery within the eggs due to deregulation of intracellular Ca^{2+} concentrations, or activation of oncosis, for instance, by massive DNA destruction leading to depletion of energy stores (Fink and Cookson, 2005). Mitochondrial disruption is also much related to this process and could be another study target to analyse whether the alteration of Ca^{2+} and pH due to OA (hypoxic conditions) may lead to fertilization failures and cell death.

To Prof Havenhand, Prof Dupont, anonymous referee 4 (p 37 li 14-25 and p38 li 1-11) about the selection of the experimental temperature for fertilization experiments and larval development

The results on early development will not be included in order to shorten the manuscript. Nevertheless we want to explain in more detail how we selected the experimental temperature.

We conducted our experiments at 3°C because this is a mean temperature from the site and the depth of collection area during the spawning period (June-August) in Kongsfjordneset. In addition we also obtained gonad samples with mature oocytes from sea urchins collected at 10-12 m depth between July-August; these individuals were at a maximal temperature in seawater of 4°C at the time of collection.

For this species there are plenty of different references about spawning season and temperature regimes (see discussion above). We based our experiments on the data of the collection period of the sea urchins used in our experiments, as there is not much information about spawning of populations at these latitudes.

To anonymous referee 4 and 6 about Results section

The suggestions were taken into account and the section had been rewritten.

To anonymous referee 4 (p 38 li 24) about language correction on the first sentences of Discussion

Cellular level refers to cytological alterations described in results. This sentence will be rephrased as:
"Our findings demonstrated significant impacts of OA on fertilization success of *S. droebachiensis* eggs at elevated seawater pCO_2 concentrations (1400 µatm and 3000 µatm)."

To Prof Havenhand (p 39 li 7) about the statement on the use of FE lifting-off as indicator of successful fertilization

The statement was rewritten as: "Successful fertilization is currently assessed in standard protocols for toxicological assays using sea urchin fertilization tests, by the presence of a well-formed and elevated FE surrounding a fertilized egg and its HL (USEPA, 1993, 2012)".

To anonymous referee 4 (p 40 li 1-2) about references for gamete contact mechanisms

The paragraph was rephrased as: "Reproduction in nature is ruled by other gamete contact mechanisms than those used in fertilization experiments, minimizing the risk of polyspermy (Himmelman et al., 2008). 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. 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 may be impared, slowed down, or even not totally completed at the time of metabolic activation, supernumerary sperms could increase the probability of polyspermic events."

To Prof Havenhand (p 42 li 15) about the inclusion of citations claiming robustness of sea urchin to OA effects

Our paragraph was rephrased as: "Within the published literature on OA effects using fertilization tests, different methodological approaches and different species have been used."

To anonymous referee 4 (p 42 li 21-29 and p43 li1-10) about reduction of the paragraph related with environmental effects

The paragraphs will be rephrased and shortened as:

"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 differences in population and species-specific responses to acidification. 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 for interpretation and comparison of results. Water parameters like salinity, alkalinity and pH may be influenced by temperature and depth (Riebesell et al., 2010), evaporation-recirculation processes due to diurnal cycles in solar radiation/seawater chemistry (Jokiel et al., 2008) and inter-decadal oscillation of pH and upwelling zones (Pelejero et al., 2005)."

To anonymous referee 4 (p 44 li 6) about the reversible effects observed in the larvae

We deleted these sentences as the data on OA effects on the larval development will be deleted from this manuscript.

To Prof Havenhand (p45 li 10-27 and p 46 li 1-5) about the discussion on the results of pH_i measurements

Between 180 and 980 µatm treatments, the cells are able to regulate the pH_i properly. At higher partial pressures, e.g. 1400 and 3000 µatm, the cells do not cope with the amount of CO_2 and are unable to keep the pH_i at physiological levels. There are two possible explanations for a similar ratio observed for 1400 and 3000 µatm. These are:

1)   The dye BCECF has a linear pH-ratio relationship between 6.4 and 7.8 pH units. If the pH is in the lower range, we do not expect to observe major ratio changes, for the curve would be at their inflexion point. The pH of the cells should have been lower than 6.5 (less probable option).

2)   The regulation of pH_i does not cope with the increased CO_2 loads and a new steady state at lower pH_i is reached and must be maintained as long as possible, before cells died. The calibration curve made in further experiments shows that there is a clear dependence of ratio from pH and therefore the technique is functioning.

To anonymous referee 4 (p 46 li 7-10) as suggested this introductory paragraph will be deleted

The first paragraph of the Discussion section will be eliminated from manuscript.

To anonymous referee 4 (p 46 li 14) about suggestion "Not supported by the data. Leave out or give reference"

The paragraph was rephrased as:

"OA impact might be stronger than expected when we consider the percentage of eggs with cellular pathologies in our fertilization tests. Although Kurihara et al. (2013) stated that in *H. pulcherrimus* the hypercapnic exposure delayed gonad maturation and spawning without having effect on the maximum number of ova spawned, several authors have reported a decrease in food intake at higher pCO_2 conditions (Siikavuopio et al., 2007; Stumpp et al.,

2012; Kurihara et al., 2013) which may affect the quality of the produced eggs. Once eggs are shed, a high percentage may face unfavourable acidic conditions impairing fertilization, metabolic activation and cellular division."

To Prof Melzner about sperm-egg communication

As we focused our study on the effects of OA on fertilization and especially on FE formation in eggs, the sperm-egg communication issue was not discussed. Nevertheless we can hypothesize that OA may affect the gamete communication either by impairing sperm activation processes and chemotaxis, or by the putative interference with the production of signalling chemoattractant peptides in the egg coat (Resact in *Arbacia punctulata* and Speract in *S. purpuratus*).

The process of sperm activation and initiation of motility starts immediately after contact with seawater, involving changes in intracellular pH in sperm cells due to a decreased CO_2 tension in seawater when compared with intra-gonadal concentrations. This change leads to a release of protons with a concomitant increase in intracellular pH (reviewed by Neill and Vacquier (2004)).

The change in internal pH is also associated to the posterior acrosome reaction once contact of gametes occurs (Lee et al., 1983). Changes in the intracellular pH to values around 7.5-7.6 are necessary for sperm activation, though below pH 7.3 the dynein ATP$_{ase}$ is inactive and respiration is inhibited (Neill and Vacquier, 2004). In human sperm the internal rise in pH is accomplished by the Hv1 voltage-gated proton channel, which contribute also in a great extent to the hyperpolarization necessary for posterior acrosomal reaction (Lishko et al., 2010).

Hyperpolarization plays a key role in capacitation and also in chemotaxis. Chemoattractants in the coat of the eggs are able to activate guanylyl-cyclase receptors in the sperm flagellum leading to hyperpolarization and activation of different voltage gated channels with a final and considerable increase in intracellular Ca^{2+} levels (Kaupp et al., 2008). This process is highly regulated and determines the swimming behaviour of the sperm upon a gradient of chemoattractant: sperms are able to register relative changes in concentration rather than absolute values to overcome saturation (Kaupp et al., 2008; Alvarez et al., 2012).

Sperm facing OA implies that the relative decrease in the CO_2 tension necessary for activation is probably lower, leading to insufficient alkalinization levels within the sperm, thus, compromising the functionality of the axoneme, chemotaxis and acrosomal reaction. On the other hand, the sperm-egg contact itself may be affected in addition by cytoskeleton alterations in the egg. Impairment of actin polymerization leading to unsuccessful formation of microvilli may interfere with the process of engulfment of the sperm.

It can be also speculated that acidification may alter chemotaxis and contact between gametes by affecting the structure of the extracellular matrix (ECM), mainly composed of fucose sulfate glycoconjugates (FSG), and the functionality of egg-jelly associated peptides (sperm activating peptides (SAP)) (Hoshino et al., 1992).

Hoshino et al. (1992) studied the effects of FSG and SAP on intracellular levels of Ca^{2+} and pH under different extracellular pH conditions (pH 8 and 6.6) and found that under acidic conditions there are no significant changes in Ca_i^{2+} by exposure to both, FSG and SAP, while during exposure to SAP only, a large increase in pH$_i$ under pH 6.6 is induced. These authors also stated that for a full induction of the acrosome reaction, SAP acts as a specific cofactor for FSG and that the observed increase in intracellular pH due to SAP is comparable to levels

necessary for stimulation of respiration in sperm. We could also hypothesize based on these results, that even when SAP seems not to be affected by acidic conditions, FSG will be impaired putatively affecting process like Ca_i^{2+} increase and elevation of cyclic nucleotides, necessary for posterior events in the acrosome reaction.

The sperm cells may suffer higher alterations in terms of cytoplasmic pH regulation due to their small size compare to eggs. In our study we did not analyse effects on sperm cells. pH measurements were made only on unfertilized eggs, therefore we cannot demonstrate on the basis of our results that the changes of the intracellular pH are directly linked to gamete contact impairments, and if alterations occurred on the ECM of unfertilized eggs, they were not evident, though no differences were observed in the morphology of the eggs between the treatments used.

Complex experiments comprising measurements of pHi after fertilization and staining of the sperm would be necessary to detect possible failures at the time of gamete contact. These could elucidate whether the eggs are able to attain the physiological pH referred for fertilized eggs in the literature after being exposed to acidic conditions. Together with the morphological alterations described in our manuscript these data could offer the link between all the affected processes.

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