Sperm motility and fertilisation success in an acidified and hypoxic environment.

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Abstract

The distribution and function of many marine species is largely determined by the effect of abiotic drivers on their reproduction and early development, including those drivers associated with elevated CO_2 and global climate change. A number of studies have therefore investigated the effects of elevated pCO_2 on a range of reproductive parameters, including sperm motility and fertilisation success. To date, most of these studies have not examined the possible synergistic effects of other abiotic drivers, such as the increased frequency of hypoxic events that are also associated with climate change. The present study is therefore novel in assessing the impact that a hypoxic event could have on reproduction in a future high CO_2 ocean. Specifically, this study assesses sperm motility and fertilisation success in the sea urchin *Paracentrotus lividus* exposed to elevated pCO_2 for 6 months. Gametes extracted from these pre acclimated individuals were subjected to hypoxic conditions simulating an hypoxic event in a future high CO_2 ocean. Sperm swimming speed increased under elevated pCO_2 and decrease under hypoxic conditions resulting in the elevated pCO_2 and hypoxic treatment being approximately equivalent to the control. There was also a combined negative effect of increased pCO_2 and hypoxia on the percentage of motile sperm. There was a significant negative effect of elevated pCO_2 on fertilisation success, and when combined with a simulated hypoxic event there was an even greater effect. This could potentially affect cohort recruitment and in turn reduce the density of this ecologically and economically important ecosystem engineer therefore potentially effecting biodiversity and ecosystem services.

Introduction

Global climate change, fuelled by enriched atmospheric carbon inventories, is altering the physicochemical status of the global ocean (Diaz and Rosenberg, 2008; Kroeker *et al*., 2010; Byrne, 2012). The increasing partial pressure of seawater CO_2 (pCO_2) is driving a

decline in seawater pH – a process termed ocean acidification (OA). Seawater pH is predicted to drop by 0.3 to 0.5 units by 2100 (based on pCO_2 concentrations of 730-1020 µatm respectively; (IPCC, 2014). The combination of rising pCO_2 and increasing sea surface temperature will place an additional burden on marine systems by reducing oxygen solubility (Hofmann and Schellnhuber, 2009). Increased frequencies of ocean hypoxic events, such as may occur *via* ocean upwelling, are predicted (Pörtner and Langenbach, 2005; Pörtner, 2008; Oschlies *et al*., 2008) making it necessary to understand the combined effects of OA and hypoxia on marine species and ecosystems (Reum *et al*., 2015).

Reproductive processes and early ontogenetic stages of marine animals appear particularly vulnerable to changing seawater properties (Pörtner and Farrell, 2008; Byrne *et al.*, 2010a,b; Cooper *et al*., 2012). Broadcast spawning, a reproductive strategy common in many marine animals, exposes gametes directly to the seawater environment (Crimaldi, 2012). Spawned gametes have therefore been used extensively in attempts to describe the potential impacts of OA on reproductive processes (Havenhand and Schlegel, 2009; Byrne *et al.*, 2010a, b; Ericson *et al.*, 2010; Frommel *et al.*, 2010; Morita *et al*., 2010; Cooper *et al*., 2012). Hitherto, reductions in seawater pH have been shown in several studies to impact sperm swimming ability by causing changes in internal pH (pH_i) of sperm and affecting motility of the flagellum (Havenhand *et al*., 2008; Fitzpatrick *et al*., 2009; Morita *et al*., 2010; Caldwell *et al*., 2011). These changes in sperm pH_i affect fertilisation by slowing the fast block to polyspermy through interfering with the Na^+/H^+ exchange and preventing the fertilisation membrane being raised (Reuter *et al*., 2011; Gonzalez-Bernat *et al*., 2013). Despite variable results, the consensus is that OA, as a function of climate change, will negatively impact marine biodiversity and ecosystem function via disruption of reproductive processes (Dupont *et al*., 2010; Byrne, 2012).

Over the past decade, the dissolved oxygen content of coastal waters has changed dramatically and this has led to widespread occurrences of hypoxia, especially in coastal areas, which have shown an exponential increase of hypoxic events of 5.54% $year^{-1}$ (Diaz and Rosenburg, 1995; Diaz, 2001; Vaquer-Sunyer and Duarte, 2008). Normal dissolved oxygen levels range between 5.0 and 8.0 mg O_2 l^{-1} in coastal waters, hypoxic conditions are defined as occurring when levels of dissolved oxygen fall below 2.8 mg O_2 l^{-1} (30 % oxygen saturation or less) (Diaz and Rosenburg, 1995). The duration of an hypoxic event can be long term/permanent, or short term (incidental, or episodic) as investigated in the present study (Middelburg & Levin, 2009). Hypoxia has been shown to negatively affect reproduction and development of marine invertebrates across a range of reproductive endpoints including gonad growth (Siikavuopio *et al*., 2007), reproduction (Cheung *et al*., 2008), egg production (Marcus *et al*., 2004), reproductive output (Spicer and El-Gamal, 1990), and embryonic development (Chan *et al*., 2008). A recent study by Shin *et al*. (2014) reported that hypoxia, as a single stressor, significantly reduced sperm motility in *Hydroides elegans*, which compromised fertilisation success. There was also a negative effect of hypoxia on embryonic development with an increase in the number of malformed embryos (Shin *et al*., 2014). As elevated pCO_2 and hypoxia, when applied individually, are reported to have similar negative effects on reproduction, they may be expected to have synergistic or additive effects when applied together. Consequently, we examined the effects of long-term exposure (6 months) of adult sea urchins to elevated pCO_2 prior to spawning, followed by the exposure of spawned gametes to hypoxia and OA before and during fertilisation. This study was designed to represent the effect of an hypoxic event in a high pCO_2 ocean, and the effects that this may have on sperm motility and fertilisation success of the sea urchin *Paracentrotus lividus*; an ecologically and economically important marine grazing species. With the occurrence of hypoxic events set to rise, it is

important to understand the potential impacts on animal reproduction in an already acidifying ocean and to consider possible effects on the future abundance and distribution of marine biodiversity.

Materials and methods

Animal husbandry and culture history

In order to assess the effects of long-term parental exposure to elevated pCO_2 on sperm motility and fertilisation success, adult *Paracentrotus lividus* (supplied by Dunmanus Seafoods Ltd, Durrus, Bantry, Co. Cork, Ireland), were exposed for six months to mean *p*CO2 conditions predicted to occur by the end of this century (Caldeira and Wickett, 2003). Exposures were conducted in the Plymouth Marine Laboratory (Plymouth, UK) Intertidal Mesocosm Acidification System (PML-IMAS) previously described by Queiros *et al*. (2014) and Collard *et al*. (2015). In brief, the nominal treatments used in the present study were 380 µatm and 750 µatm pCO_2. Within each of these nominal treatments urchins were randomly assigned to one of four tanks per pCO_2 treatment (8 tanks total, tank volume 1 m^3). Within each of these separate tanks, urchins were further divided into three baskets (30 cm x 20 cm x 20 cm) with original stocking densities of six urchins per basket (18 per tank). The temperature of each tank was maintained independently using aquarium heaters (Aqua One, 150W, Kong's (UK) Limited, Romsey, UK.) and chillers (BOYU L-350). pCO_2 gas mixes were also supplied separately to each tank.. Ambient pCO_2 treatments were maintained by bubbling untreated air through the water in each tank. Elevated pCO_2 treatments were maintained by enriching the air with CO_2 before bubbling (after Findlay *et al*., 2008). pCO_2 levels (µatm) of both the untreated and CO_2 enriched air were monitored using a CO_2 Analyser (LI-820, Li-Cor , Lincoln, USA) . To prevent pCO_2

and temperature gradients forming within the tanks, the water was circulated using pumps (Aquael 1000 filter, Aquael, Warszawa, Poland). Natural seasonal variation in temperature and photoperiod is known to impact on gametogenesis and spawning condition. Consequently, these cycles were recreated in the laboratory by monthly adjustments in temperature appropriate to replicate the mean ambient monthly seasonal temperature of Plymouth Sound. Photoperiod was also adjusted monthly by changing the length of time the lighting was on each day using T8 triphosphor fluorescent tubes (which are designed to meet saltwater aquarium lighting requirements) to match natural seasonal changes in day length. Each tank ($1m^3$) received a one-third by volume water change every three weeks or if nitrate levels, which were monitored weekly using a nutrient autoanalyser (Branne and Luebbe Ltd. AAIII; Brewer and Riley, 1965), exceeded 25 mg L^{-1}, however no particular tank needed to changed more often than others. Urchins were fed *ad libitum* for 48 h once every week with fresh macroalgae (*Ulva lactuca* and *Laminaria* sp; approx. 500 g per basket) collected from Plymouth Sound. Following feeding, the remaining macroalgae and any faecal pellets were removed to prevent nitrate build up.

Simulated hypoxic events

After six months of acclimation to present ambient and future predicted pCO_2 levels in the PML-IMAS, 20 randomly selected adult *Paracentrotus lividus* (7 from the 380 µatm treatment and 13 from the 750 µatm treatment) were induced to spawn by intra-coelomic injection of 0.5 - 1.0 mL 0.5 M KCl until gametes from 3 males and 3 females from each treatment had be collected for analysis for fertilisation success (below). Three males were used for sperm motility analysis and at least 200 sperm were tracked per time point per individual Subsamples of the gametes collected from these individuals were then

exposed to either normoxic or hypoxic conditions at their respective acclamatory pCO_2 level. Oxygen content was manipulated through input of nitrogen into sealed chambers, Normoxic conditions were set at >80 % dissolved oxygen (DO) and hypoxic conditions were maintained at <30% DO. Normoxic or hypoxic air from these chambers was then mixed with CO_2 in a second sealed chamber to produce either 380 or 750 µatm pCO_2 and monitored using a CO2 analyser (LI-820, Li-Cor , Lincoln, USA) before entering the experimental chambers where the well plates containing the sperm motility and fertilisation assay were placed. Oxygen content in these plates was determined using an OxySense® system (OxySense® 5250i, Dallas, USA) for both normoxic and hypoxic conditions. pH was monitored continually using a micro pH probe (Micro-Inlab pH combination electrode, Metter Toledo, Leicester, UK) connected to a calibrated pH meter (Seven Easy pH meter, Metter Toledo, Leicester, UK)).. . Temperature was maintained to match the monthly acclimation temperature of 15 °C using a water bath (Grant Cambridge Ltd, Cambridge, UK) and was monitored using a K-type thermocouple in each chamber connected to a temperature logger (Omega, HH806AU, Manchester, UK). Specific water chemistry for gametes incubations are shown in table 2.

Reproduction analysis

Following spawning (as described above), sperm were collected dry (i.e. undiluted) and stored on ice for no more than 1 h. Sperm were not pooled and males were treated as individuals. Female were allowed to express their eggs for 1 h, and the eggs kept separate for analysis. Egg densities were determined by counting 3 x 50 µL aliquots from each female. Sperm densities were determined by haemocytometer and adjusted to 10^7 sperm ml^{-1} using either hypoxic or normoxic filtered sea water at 380 or 750 µatm pCO_2 (FSW

0.22 µm filtered). Three Subsamples (5 µL) of sperm from each individual, held at 15 °C from each of the combined CO_2 and oxygen levels (380 µatm pCO_2 and 750 µatm pCO_2; 30 % and >80 % O_2 saturation; table 2) were taken at 10 minute intervals (from 1 to 61 minutes) and transferred immediately to a glass slide (18 samples per individual in total). Sperm motility, determined as percentage motility and swimming speed (curvilinear velocity, VCL) was measured by computer assisted sperm analysis (CASA) at 15 ^{o}C according to Caldwell *et al*. (2011). A minimum of 200 sperm were tracked per time point.

Fertilisation assays were conducted at combined CO_2 and oxygen levels (380 µatm and 750 µatm; 30 % and >80 % O_2 saturation; table 2) in 6-well multi-plates with gametes collected from 3 males and 3 females at densities of 2.5×10^5 ml^{-1} for sperm and 500 eggs per well, containing 10 ml FSW. Fertilisation success was determined after two hours based on the occurrence of the first mitotic cleavage.

Carbonate chemistry

Seawater for the experimental system was collected from PML's long term monitoring site, the Western Channel Observatory, station L4 ('50º 15.00'N, 4º 13.02'W). The physico-chemical parameters (temperature, salinity, pH, dissolved inorganic carbon (DIC), and total alkalinity (A_T)) of the seawater were measured three times a week for the duration of the experimental period using the methods of Findlay *et al.* (2013). Additional carbonate system parameters were calculated from temperature, salinity, A_T and pH as described in Findlay *et al.* (2013). The long-term physico-chemical data are presented in Findlay *et al.* (2013) and Collard *et al*. (2015). The water chemistry parameters after six months of incubation in the 380 µatm and 750 µatm ambient temperature treatments used

199 in the present study are presented in Table 1. The physico-chemical parameters for gamete
200 incubation were measured/calculated in the same way and are presented in table 2.
201
202 *Data analysis*
203
204 Motility data from sperm with a head area <5 μm^2 and >35 μm^2 were discounted to
205 eliminate false negatives attributable to sperm clumping or sperm misidentification by the
206 CASA software (Caldwell *et al*., 2011). A test for normality (Kolmogorov-Smirnov) was
207 carried out and data transformed using a natural log when not normally distributed. A 2-
208 way ANOVA was conducted on the log VCL data to determine significant factors and
209 interactions using time as a co-factor. Percentage sperm motility and fertilisation success
210 data were arcsine transformed prior to statistical analysis and a test for normality
211 (Kolmogorov-Smirnov) was carried out. Two-way ANOVA was conducted, for percentage
212 sperm motility time was used as a co-factor.
213
214 **Results**
215
216 *Sperm motility*
217
218 Neither time ($p = 0.141$) nor pCO_2 ($p = 0.370$) as single variables significantly affected
219 percentage motility (Table 3 a, Fig. 1a). Percentage motility decreased under hypoxia at
220 both 380 μatm pCO_2 ($p = 0.032$) and 750 μatm pCO_2 ($p < 0.005$; Table 3 a) levels.
221 Hypoxia at 750 μatm pCO_2 led to the lowest percentage motility, although this did not
222 differ significantly from the percentage motility at the 380 μatm pCO_2 hypoxic level; and
223 there was no significant interaction between pCO_2 and hypoxia (Tabel 3 a; Figure 1a).

Swimming speed (VCL) increased at 750 µatm pCO_2 under both normoxic and hypoxic conditions relative to 380 µatm pCO_2 treatments (Table 3 b, Figure 1 b). Both pCO_2 and hypoxia separately showed significant effects on VCL (both $p < 0.01$), however there was no significant interaction (Table 3b). VCL was significantly reduced under 380 µatm pCO_2 hypoxic conditions ($p<0.05$) compared with controls. Overall there was a significant effect of time on VCL ($p < 0.01$) (Figure 2). This was driven by changes in the 380 µatm pCO_2 and 750 µatm pCO_2 normoxic treatments. In the 380 µatm pCO_2 normoxic treatment VCL was highest at 1 minute and significantly decreased after 50 minutes ($p<0.05$) and 60 minutes ($p<0.05$); figure 2). In the 750 µatm pCO_2 normoxic treatment VCL was highest at 10 minutes this significantly decreased at 20 minutes ($p<0.01$). Although this decrease did not remain significant at 30 minutes ($p=0.115$) and 40 minutes ($p=0.051$) it was significant at 50 minutes ($p<0.01$) and 60 minutes ($p<0.01$; Figure 2). There was no significant difference in VCL across track time in the 380 µatm pCO_2 ($p= 0.844$) and 750 µatm pCO_2 ($p=0.719$) hypoxic treatments.

Fertilisation success

Fertilisation success was significantly reduced by both elevated pCO_2 and by reduced oxygen. Under normoxic conditions, the elevated pCO_2 caused a decrease of 7% ($p = <0.005$). Hypoxic conditions under normal pCO_2 levels, however, caused a further decrease by 63% ($p = <0.005$). The combined impact of high pCO_2 and low oxygen was most detrimental, with fertilisation success reduced to 3% ($p = <0.005$). There was, therefore, a significant interaction between hypoxia and elevated pCO_2 ($p = <0.005$).

Discussion

249

250 The results of the current study suggest that if an hypoxic event were to occur
251 under future ocean acidification scenarios, there would be a significant decrease in the
252 fertilisation success of *P. lividus*, although sperm motility would not be significantly
253 affected by combined pCO_2 and hypoxic conditions. The results also highlight the need
254 for further studies into the synergistic effects of abiotic factors, as ocean acidification is
255 unlikely to occur in isolation from other climate related stressors such as warming and
256 hypoxia.

257 There was no significant effect of pCO_2 on the percentage of motile sperm, in
258 agreement with a previous study (Havenhand and Schlegel, 2009). Although sperm
259 swimming speed, which remained high across all treatments, was significantly higher at
260 elevated pCO_2. In contrast, the majority of previous studies (e.g. Havenhand and Schlegel,
261 2009; Frommel *et al*., 2010; Morita *et al.,* 2010) concerned with sperm swimming speed
262 reported a slowing under acidified conditions. However the current study differs from
263 much of the previous literature as sperm motility and swimming speed were tracked over a
264 one-hour period; substantially longer than many previous studies which have used track
265 times of a few seconds post activation (Morita *et al*., 2010; Schlegel *et al*., 2012). This
266 longer tracking time was used, because fertilisation of broadcast spawners may not
267 necessarily happen immediately, as gametes need to disperse. Tracking for one hour
268 allows a more realistic assessment of what may happen naturally. Consistent with this
269 reasoning, the present study shows that changes in sperm swimming speed over the first
270 hour of activation differed between treatments (Figure 2); a point which may have been
271 missed previously due to shorter tracking times. An explanation for an increase in sperm
272 swimming speed is offered in previous work (Caldwell *et al*., 2011) by means of sperm
273 activation pH. This is the mechanism whereby sperm are stored in an immotile state at pH

7.2, below the activation threshold of sperm dynein ATPase that powers the flagellum (Johnson *et al*., 1983). When the sperm are released into the water column the pH of the sperm is increased to 7.6 and the flagellum is activated and mitochondrial respiration begins (Christen *et al*., 1983). This indicates that there will be an increase in sperm swimming speed, perhaps modulated by sperm-activating peptides (SAPs), which are released by the egg jelly coat. These SAPs evolved 70 million years ago when atmospheric CO_2 was far higher than present day levels and oceans had a lower pH (pH 7.4-7.6) (Neill and Vacquier, 2004; Darszon *et al*., 2008; Caldwell *et al*., 2011).

Hypoxia is also an important factor in relation to sperm motility. The current study shows that both sperm percentage motility and VCL were reduced under hypoxic conditions. Previous research into the effects of hypoxia on sperm swimming speed gave contrasting results, with the majority of studies seeing a reduction in sperm swimming speed when exposed to hypoxic conditions (Bencic *et al*., 1999a, b; Wu *et al*., 2003; Shin *et al*., 2014) similar to the results described here. Sperm motility is an energetically demanding process requiring ATP, which is generated in mitochondria located in the mid piece of the sperm. In the absence of oxygen ATP cannot be synthesised from ADP *via* oxidative phosphorylation, thereby limiting energy availability for flagellum activity. Therefore, under hypoxic conditions where oxygen availability is limited, sperm are unable to become active (Billard and Cosson, 1990; Fitzpatrick *et al*., 2009). However when increased pCO_2 and hypoxia are considered together, both percentage sperm motility and sperm VCL did not differ significantly from the control treatment. If the reduction in sperm motility through hypoxia is considered with the increase in sperm swimming speed due to increasing pCO_2, there is potential for a mediating effect of hypoxia on the impact of OA.

In contrast to sperm motility, fertilisation success is reduced under both increased pCO_2 and hypoxic conditions. The effects of increased pCO_2 on fertilisation success have been widely studied and are believed to be attributable to developmental delay (Kurihara and Shiryiama 2004) or to the slowing of the fast block to polyspermy (Reuter *et al*., 2011). Previous studies on the effects of OA on fertilisation success have obtained variable results but there was no significant effect on fertilisation success in the majority of studies on echinoderms (e.g. Byrne *et al*., 2009; Byrne *et al*., 2010a, b; Martin *et al*., 2011). However, a few studies have obtained results similar to those of the current study. A reduction in fertilisation success under OA was noted for the sea urchins *Paracentrotus lividus* (Moulin *et al*., 2011) and *Heliocidaris erythrogamma* (Havenhand *et al*., 2008). These intra- and inter-specific differences have previously been attributed to variations in experimental design. In addition contrasting with the present study none of these previous studies have pre acclimated the adults from which the gametes were obtained.

Here, hypoxia as a single factor caused a significant decrease in fertilisation success; in general studies on effects of hypoxia on reproductive capacity show a significant negative effect on reproductive endpoints including fertilisation success. This significant reduction suggests that early embryonic development is reliant on aerobic respiration. Respiratory rate in sea urchin eggs has previously shown a marked increase after fertilisation (Yasumasu *et al*., 1996), which would account for the reduction seen here under hypoxic conditions. After fertilisation, oxygen is required primarily for the oxygenation of glycogen, which is stored in the eggs and is an essential energy reserve for development. The oxygen used is attained through diffusion across the oocyte membrane and this diffusion is determined by the difference in oxygen partial pressure between the egg and the external environment. For broadcast spawners, the relevant conditions are those of the external marine environment (Herreid, 1980; Wang and Zhan, 1995). Hypoxic

conditions may cause a decrease in this gradient, thus the eggs are less capable of acquiring adequate oxygen, which in turn may lead to the inhibition of embryonic development. Riveros *et al*. (1996) showed a significant reduction in fertilisation success (below 40%) when the sea urchin *Arbacia spatuligera* was exposed to oxygen levels of 30% and below. Similarly, in the sea urchin *Strongylocentrotus droebachiensis* there was a significant negative effect of hypoxia on gonad growth (Siikavuopio *et al*., 2007). Reductions in reproductive ability and output also occur in brine shrimp (Spicer and El-Gamal, 1990), copepods (Marcus *et al*., 2004; Sedlaceck and Marcus, 2005; McAllen and Brennan, 2009) and gastropods (Cheung *et al*., 2008). The results from previous studies also indicate a reduction in energy allocation for reproduction (Cheung *et al*., 2008) as well as a reduction in developmental rate, indicating developmental delay (McAllen and Brennan, 2009).

The results of the present study suggest a synergistic effect between increased pCO_2 and hypoxia, as there was a significant reduction in fertilisation success under hypoxic conditions and a significant difference between the 380 μatm and 750 μatm treatments. The diffusion of pCO_2 created during respiration is reliant on a diffusion gradient similar to that for oxygen and under increased pCO_2 the CO_2 molecules do not move as readily across the egg membrane, leading to reductions in fertilisation success. This synergistic effect may lead to severe negative effects on species recruitment and distribution. Recent studies (Gobler *et al*., 2014) also found a negative synergistic effect of increasing OA and hypoxia in relation to larval development and survivorship. Reduced survivorship (by >50 %) and inhibition of growth and metamorphosis (by >50 %) occurred under low oxygen conditions in two calcifying bivalves: bay scallops, *Argopecten irradians*, and hard clams, *Mercenaria mercenaria*. However, in contrast to Gobler *et al*. (2014), Frieder *et al*. (2014) found that there was no significant effect of low pH or low O_2

on survivorship of the mytilid species, *Mytilus californianus* and *M. galloprovincialis*, and no effect of combined increased pCO_2 and low O_2 on their early development.

The present study is novel in assessing the impact that an hypoxic event would have on the reproductive parameters of sperm motility and fertilisation success in a future high CO_2 world. There is a significant effect of both pCO_2 and hypoxia on sperm swimming speed, with reduced speeds being seen under hypoxic conditions and increased speeds being seen under increased pCO_2 levels. In normoxic conditions increased speed at elevated pCO_2 could possibly have negative effects on fertilisation success because sperm that swim faster use up their available energy faster (motility decreased after 20 minutes) leading to a possible trade-off between sperm speed and longevity. This suggests that sperm swimming speed is not necessarily the most important factor in fertilisation success. Broadcast spawning is affected by many factors, including water currents and chemistry, and as fertilisation may not happen immediately, sperm need to be motile for longer (Levitan, 2000) and so sperm released in a future high pCO_2 ocean may use up their energy quicker and result in a reduction in fertilisation success. However as also shown in this study, if swimming speed decreases to lower levels, as associated with hypoxia under ambient pCO_2 levels, fertilisation success is reduced despite swimming activity remaining constant for longer (> 1 hour). In addition, when elevated pCO_2 and hypoxia are combined their contrasting effects lead to sperm swimming speed similar to that observed in control treatments and swimming activity remaining constant for over an hour. Despite this fertilisation success was lowest in this treatment. This suggests that a least under the combined of hypoxia and elevated pCO_2 the direct synergistic effects of these stressors on fertilisation success is more important than indirect effects of sperm motility and longevity.

It appears that an hypoxic event will negatively affect fertilisation success regardless of oceanic pCO_2, but this effect will be intensified under near future pCO_2 conditions. This is in contrast to the results for sperm motility which suggests an increase in sperm swimming speed under increased pCO_2 conditions which will be mediated by a hypoxic event. If fertilisation success is negatively impacted, there will likely be knock-on effects such as reduced recruitment but also effects on the food chain, as *P. lividus* is not only an important grazing species but also an important source of prey for larger organisms. It is also an important commercial species and the impacts of climate change may negatively affect its aquaculture.

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589

590 **Table 1.** Water chemistry parameters for the ambient and future predicted OA scenarios
591 (after Findlay *et al*., 2013). Parameters labelled with * were calculated using CO2Sys
592 software. Seasonal light(L):dark(D) cycles are presented for the date of the experiment.

Nominal pCO_2 treatment (µatm)	**380**	**750**
TA (µmol kg^{-1})	2255.24 ± 133.1	2183.17 ± 101.6
pH	8.08 ± 0.03	7.93 ± 0.09
Temperature (ºC)	15.04 ± 0.90	15.66 ± 0.65
Salinity	35.00 ± 0.1	34.90 ± 0.2
DIC (µmol kg^{-1})*	2073.90 ± 122.9	2062.90 ± 131.3
pCO_2 (µatm)*	483.00 ± 24.6	722.40 ± 198.2
Ω Cal*	3.18 ± 0.25	2.31 ± 0.32
Ω Arg*	2.04 ± 0.15	1.49 ± 0.21
L:D cycle	16:8	16:8
Nitrate	7.313±12.97	7.93±13.20

593

594 **Table 2**. Water Chemistry parameters for the ambint and future predicted OA scenarios
595 used in experimental chambers. Parameters labelled with * were calculated using CO2Sys
596 software.

597

Nominal oxygen and pCO_2 treatment (µatm)	**380 normoxic**	**380 hypoxic**	**750 normoxic**	**750 hypoxic**
Sperm motility				
TA (µmol kg^{-1})	2366.70±68.3	2366.70±68.3	2207.05±222.5	2207.05±222.5
pH	8.07 ±0.01	8.07±0.01	7.94±0.01	7.94±0.01
Temperature (ºC)	15.2±0.12	15.3±0. 07	15.3±0. 09	15.24±0.08
Salinity	35.0±0.1	35.0±0.01	34.9±0.2	34.9±0.2
DIC (µmol kg^{-1})*	2123.90±3.13	2036.72±2.90	2125.73±3.28	2036.31±3.30.
pCO_2 (µatm)*	376.36±5.54	501.89±8.89	382.29±6.27	499.99±11.97
Ω Cal*	4.17±0.05	3.00±0.004	4.14±0.04	3.00±0.05
Ω Arg*	2.68±0.03	1.93±0.03	2.66±0.03	1.93±0.03
Oxygen (µatm)	190.70±4.4	54.08±3.0	189.85±4.2	52.96±3.1
Fertilisation				
TA (µmol kg^{-1})	2366.70±68.3	2366.70±68.3	2207.05±222.5	2207.05±222.5
pH	8.08±0.01	8.06±0.02	7.94±0.01	7.95±0.02
Temperature (ºC)	15.2±0.16	15.3±0.08	15.3±0.17	15.3±0.08
Salinity	35.0±0.1	35.0±14	34.9±0.2	34.9±0.2
DIC (µmol kg^{-1})*	2122.45±5.90	2035.92±4.58	2130.62±9.42	2031.3±10.16

pCO_2 (µatm) *	375.88±11.37	498.14±13.96	390.63±18.75	486.41±29.72
Ω Cal*	4.19±0.09	3.01±0.06	4.07±0.09	3.07±0.15
Ω Arg*	2.69±0.06	1.94±0.04	2.61±0.09	1.98±0.09
Oxygen (µatm)	190.96±3.6	52.11±4.8	190.81±3.7	53.24±4.4

598

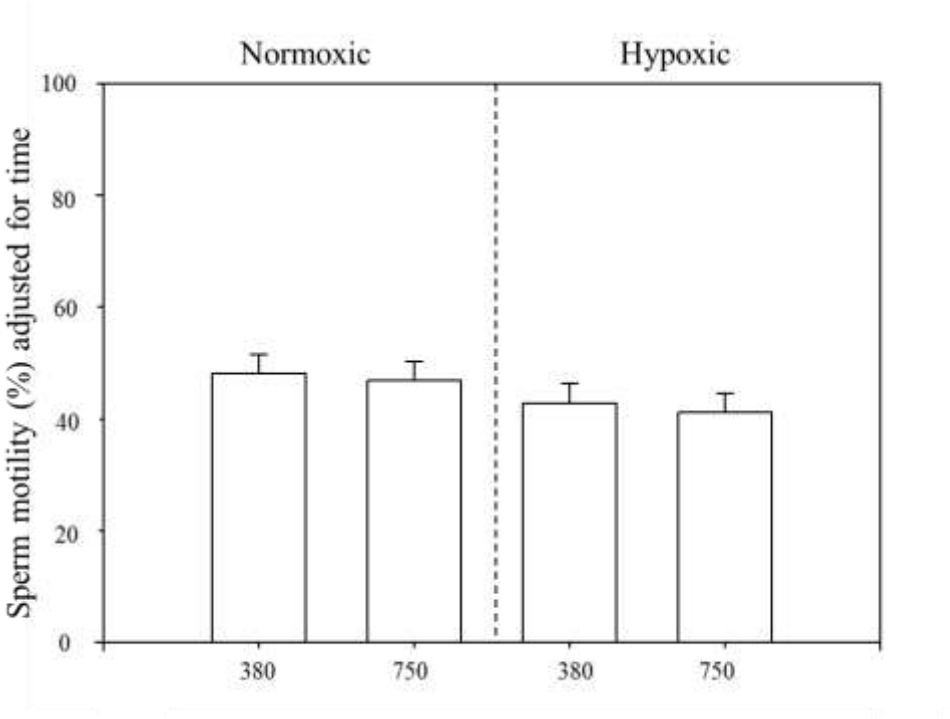
599 Table 3. ANOVA table for (*a*) percentage sperm motility; (*b*) sperm curvilinear velocity;
600 and (*c*) fertilisation success at elevated pCO_2 (750 versus 380 µatm) in combination with
601 hypoxic and normoxic conditions. Sperm motility data corrected for time.

	Df	Sum squared	Mean squared	F-Value	P(>f)
(*a*)					
pCO_2	2	49.961	49.961	0.813	0.370
Oxygen	2	643.293	643.293	10.470	0.002
Time	7	135.788	135.788	2.210	0.141
pCO_2*Oxygen	4	1.023	1.023	0.017	0.898
residuals	79	4853.940	61.442		
(*b*)					
pCO_2	2	3.253	3.253	9.105	0.003
Oxygen	2	3.445	3.445	9.642	0.003
Time	7	4.013	4.013	11.233	0.001
pCO_2*Oxygen	4	0.069	0.069	0.194	0.661
residuals	79	28.223	0.357		
(*c*)					
pCO_2	1	1621.303	1621.303	62.735	<0.005
Oxygen	1	20801.082	20801.082	804.876	<0.005
pCO_2*Oxygen	1	521.013	521.013	20.160	<0.005

residuals	32	827.002	25.844

602

(*a*)



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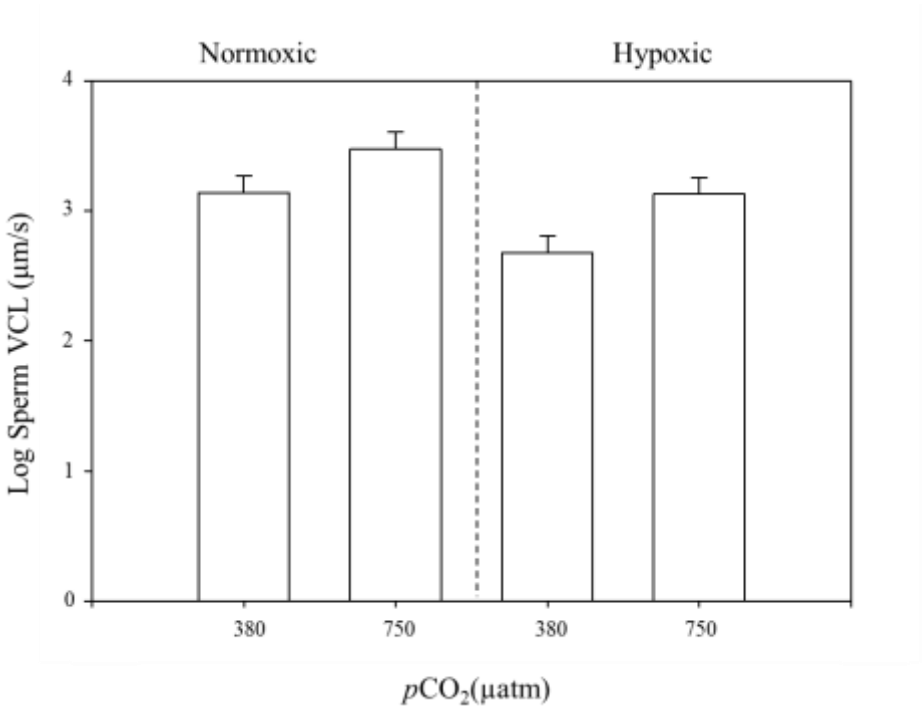


604

605 Figure 1. The effects of CO_2-induced acidification in combination with hypoxia on

606 *Paracentrotus lividus* sperm: (*a*) percentage sperm motility adjusted for time and (*b*) log

VCL. Means ± 95 % confidence intervals. Graphs show estimated marginal means. Graph (*b)* adjusted for time at 30 minutes.

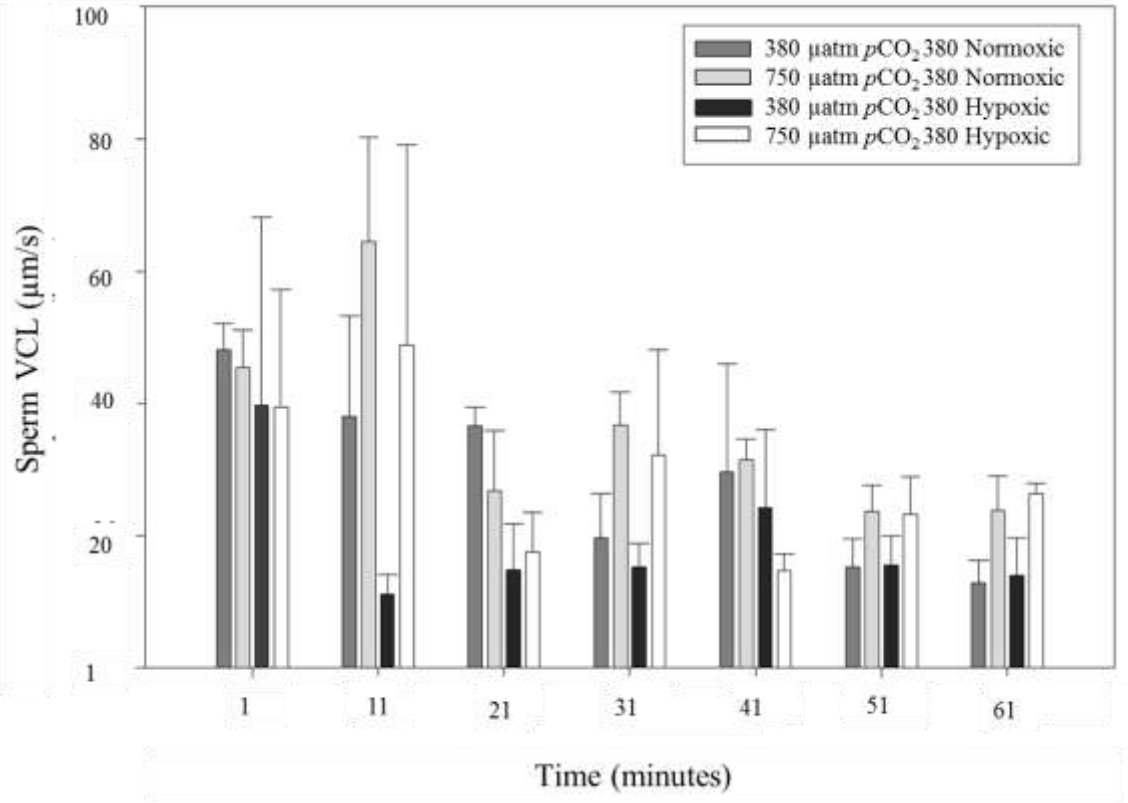
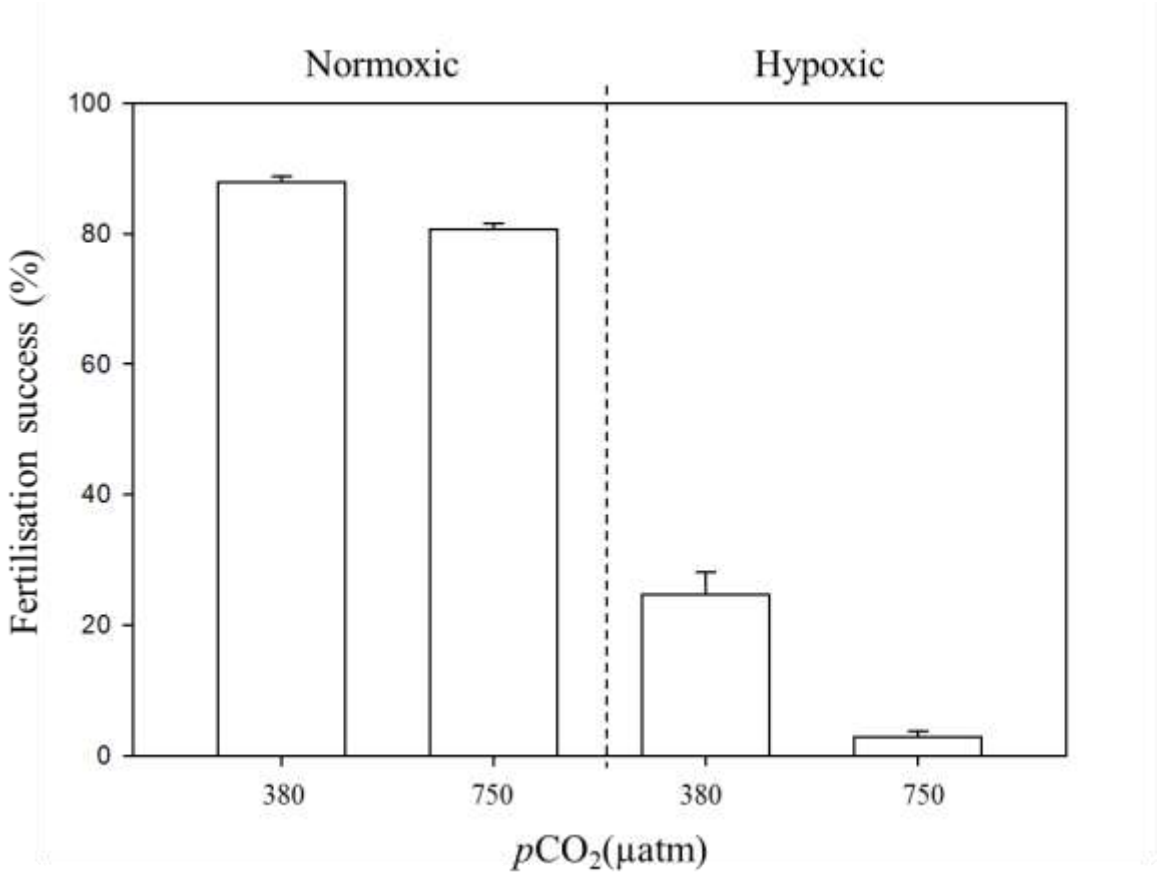


Figure 2: The effects of CO_2-induced acidification in combination with hypoxia on *Paracentrotus lividus* sperm swimming speed (VCL) over time. Data are means ±95% confidence intervals.



621

622 Figure 3. Effects of CO_2-induced acidification in combination with hypoxia on

623 *Paracentrotus lividus* fertilisation success. Data are means ± 95 % confidence intervals.

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625

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