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Near-future levels of ocean acidification do not affect sperm motility and fertilization kinetics in the oyster *Crassostrea gigas*

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Abstract

An increasing number of studies are now reporting the effects of ocean acidification on a broad range of marine species, processes and systems. Many of these are investigating the sensitive early life-history stages that several major reviews have highlighted as being potentially most susceptible to ocean acidification. Nonetheless there remain few investigations of the effects of ocean acidification on the very earliest, and critical, process of fertilization, and still fewer that have investigated levels of ocean acidification relevant for the coming century. Here we report the effects of near-future levels of ocean acidification (≈−0.35 pH unit change) on sperm swimming speed, sperm motility, and fertilization kinetics in a population of the Pacific oyster *Crassostrea gigas* from western Sweden. We found no significant effect of ocean acidification – a result that was well supported by power analysis. Similar findings from Japan suggest that this may be a globally robust result, and we emphasise the need for experiments on multiple populations from throughout a species' range. We also discuss the importance of sound experimental design and power analysis in accurate interpretation of non-significant results.

1 Introduction

It is now accepted that increasing atmospheric CO_2 is causing reductions in oceanic pH – a process widely referred to as Ocean Acidification ("OA"). Global ocean pH has fallen by an average of 0.1 pH units since the onset of the industrial revolution and several estimates show that oceanic pH could fall additionally by ≤0.4 pH units by the year 2100 (Caldeira and Wickett, 2003; Raven et al., 2005; Blackford and Gilbert, 2007; IPCC, 2007). This results in decreases in the saturation states of calcite and aragonite, the two common crystalline forms of biogenic $CaCO_3$. Estimates of future rates of reduction in saturation states vary, but it has been predicted that high latitude oceans will become undersaturated with respect to aragonite (the more soluble of the

BGD

6, 4573–4586, 2009

two forms) by the year 2050 (Orr et al., 2005). Extensive field observations from the US west coast have already found seasonal upwelling of undersaturated waters reaching the surface (Feely et al., 2008). The extent of this process in other parts of the world is currently unknown.

The biological consequences of these changes have been reported in several recent studies (see reviews by Harley et al., 2006; Fabry et al., 2008; Widdicombe and Spicer, 2008; Doney et al., 2008). These studies show that if ocean pH does fall by a further 0.4 units, there will likely be substantial negative effects on calcification and physiological processes in a wide variety of species and ecosystems. These effects are particularly relevant to the very earliest life-history stages of marine invertebrates, fertilization, embryogenesis and larval development, which are not only often the most sensitive life-stage to environmental change, but which are also key for the successful recruitment – and hence survival – of the species (Pechenik, 1999; Cowen et al., 2000; Raven et al., 2005).

Many workers have investigated the impacts of mineral acids on gametes and fertilization in marine invertebrates, but the effects of these acids are very different to those of CO_2-induced acidification (Kurihara and Shirayama, 2004; Kurihara, 2008). Relatively few studies have investigated the impacts of CO_2-induced changes in pH on fertilization (reviewed by Kurihara, 2008). Kurihara and Shirayama (2004) studied the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* finding that fertilization success declined with pH, and was statistically significant at 5000 ppm CO_2 ($\approx$pH$\leq$7.1). Havenhand et al. (2008) studying the urchin *Heliocidaris erythrogramma* found statistically significant reductions in fertilization success at pH 7.7 ($\approx$1000 ppm CO_2). Lastly, Kurihara et al. (2007, 2009) found no significant effect of 2000 ppm CO_2 ($\approx$pH 7.4) treatments on fertilization success of the bivalves *Crassostrea gigas* and *Mytilus galloprovincialis* from Japan (although there were significant subsequent effects on larval development – see below).

Substantial intraspecific variation in response of fertilization success to OA was also found by Kurihara and Shirayama (2004), perhaps indicating the capacity for heritable

variation within populations (Kurihara, 2008). Variable fertlization success can be a result of variable gamete quality, but is more commonly a natural consequence of differential compatibilities of male and female gametes: put simply, some gametes are more compatible and fertilize more easily at a given sperm concentration (e.g. Styan et al., 2008). Compatibility is most easily determined using fertilization kinetics curves, which fit a first-principles model of fertilization kinetics to fertilization success data from multiple sperm concentrations (Vogel et al., 1982; Styan, 1998; Styan and Butler, 2000). This model incorporates both concentration and swimming speed of sperm as key determinants of fertilization success.

In the only study to date that has investigated the impacts of OA on fertilization kinetics, Havenhand et al. (2008) found statistically significant changes in sperm swimming speed and percent motility that, when translated into the fertilization kinetics model, predicted a 24.9% decrease in fertilization success. This corresponded closely with the 20.4–25.9% decreases in fertilization success they observed in their experiments. They concluded that, for the urchin species they studied, OA-induced reductions in fertilization success were a result of the impacts of OA on sperm swimming behaviour.

More recent work (Havenhand, Renborg, Williamson, Mifsud, unpublished) has shown that impacts of OA on fertilization success vary markedly, even between closely related species. Here we report an investigation of the impacts of CO_2-induced ocean acidification on the sperm swimming behaviour and fertilization kinetics of the Pacific oyster *Crassostrea gigas*. We also provide statistical power analyses of non-significant results to address the key question of whether there were no biologically significant effects of pH, or whether there were significant effects but that our experimental design was unable to detect these.

2 Methods

2.1 General methods

Individual *C. gigas* were collected on multiple occasions between 11 July 2008 and 6 August 2008 from a mixed mussel/oyster bed (58°52.1′ N, 11°09.4′ E) close to the Tjärnö Marine Biological Laboratory, western Sweden. Individuals were held in flow-through surface sea water (mean T°±s.e.=20.1±0.46°C, salinity=24.0±0.39‰, total alkalinity=2.02 (measured at 5 m depth at SMHI station, Släggö)). Oysters were fed daily *ad libitum* with a mixture of microalgae (Shellfish Diet 1800 from Instant Algae©).

This same seawater, filtered to 0.22 µm, (FSW, mean pH 8.15) was used throughout for experiments. Acidified FSW was created by bubbling CO_2 until a stable pH reduction of ~0.35 units from FSW pH had been obtained (Table 1). Bubbling was monitored manually and pH was measured using a calibrated pH-meter (pH-212 Hanna instruments). FSW was used as a control medium in experiments.

Oysters were strip-spawned by drilling a hole through the shell above the gonads and pipetting out dry sperm and eggs. Sperm were kept "dry" on ice to extend their lifespan. Eggs were held in FSW in a Petri-dish for ~30 min before use. Experiments were conducted at 21–22°C as soon as possible after spawning.

Sperm suspensions were generated by diluting 1–5 µl dry sperm (depending on concentration) from a single male oyster into 1–2 ml of pH 8.15 or pH 7.8 FSW immediately before use. Dilutions generated 10^3–10^4 sperm μl^{-1} which prior experiments had shown to be optimal for sperm motility assessment. A small drop (≈100 µl) of this sperm suspension was placed on an albumin-coated microscope slide and a coverslip, which were separated by a 0.75 mm thick O-ring to minimise wall effects on sperm swimming speed (Havenhand et al., 2008). Sperm behaviour was then recorded onto DV-tape at 25 frames s^{-1} using an inverted compound microscope (Leica© DM-IL, 10× objective) and digital video camera (Sony 3CCD ExwaveHD). All recordings were made within 10 s of the sperm suspension being placed on the slide. Preliminary trials

BGD
6, 4573–4586, 2009

showed that the pH of this drop of sperm suspension did not significantly change over this period: mean ΔpH± s.e. after 100 s was −0.010±0.005 pH units. Ten replicate observations (slides) were made for 10 individually prepared sperm suspensions in each of pH 8.15 and pH 7.8 for each male. Video was post-processed with Final Cut Pro© (Apple Computer, Cupertino, CA), and 1 s video clips from each slide (replicate) were analyzed using CellTrak1.3© (Motion Analysis Corporation, Santa Rosa, CA). Average sperm speed and percentage of motile sperm were determined for each slide. This process was repeated for each of 14 different males.

For fertilization success experiments, eggs were extracted from three or more female oysters and mixed with sperm from one male (where possible these were the same males for which motility data were obtained; Tables 1 and 2). Mixed batches of eggs were used to minimize sperm-egg incompatibilities. Fertilization protocols followed those used by Havenhand et al. (2008). Briefly, fertilizations were carried out in filter-dishes (25 mmØ×20 mm H, 20 µm mesh floor) placed in each well of two 6-well plates. Wells were filled with 5 ml of FSW or acidified FSW (Table 1). For each pH, a 6-step, 6-fold sperm dilution series was created. Sperm concentrations were checked *post-hoc* by haemocytometer counts. An aliquot of 50 µl of egg suspension (500–2000 eggs, depending on concentration) was added to each filter dish and then transferred to the sperm suspension and left for 12 min. Sperm were then removed from the eggs by rinsing the filter dishes with FSW or acidified FSW. Eggs were then transferred to new 6-well plates containing FSW or acidified FSW. Fertilization success rate (percentage of fertilized eggs) was determined after ~1 h by directly enumerating the proportion of 200 eggs that showed a polar-body or cleavage.

2.2 Data analysis

Before statistical analysis all percentage data were arc-sin transformed to meet the assumption of normality, which was subsequently checked by inspection of box-plots (Quinn and Keough, 2002). The assumption of homogeneity of variance among sub-

groups (different males, treatments, etc.) was assessed using Levene's test. No significant differences among variances of subgroups were found.

Effects of pH on swimming speed and percent motility of sperm for individuals males were assessed by *t*-test using SPSS™. Effects of pH on swimming speed and percent motility of sperm across all males were assessed by two-factor mixed-model ANOVA (pH=fixed, date/male=random), again using SPSS™. The power of our experiments (the likelihood that our tests would have detected a biologically meaningful effect had it existed) was determined by power analysis using the program G*Power (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/).

3 Results

Mean sperm swimming speeds were very similar at both pH levels (mean ±s.e. for pH 8.15=92.1±4.8 µm s^{-1}, pH 7.8=94.3±5.5 µm s^{-1}, Table 1). Similarly, there was a very small effect of pH on mean percent motility of sperm (mean ±s.e. for pH 8.15=55.3±3.7% and for pH 7.8=54.9±3.8%, Table 1). These differences were not statistically significant (ANOVA sperm speed, $F_{1,15}$=0.911, P=0.355; percent motility, $F_{1,15}$=0.376, P=0.549). Power analysis showed that these tests had >80% power to detect a 5% change in percent motility.

Highly significant positive and negative effects of pH on sperm swimming speed were observed in some individual males, and two significant effects on sperm motility were observed (*t*-test, Table 1). Most males showed non-significant results. Power analyses were not conducted for individual males.

Mean fertilization success in pH 8.15 was similar to that in pH 7.8 (63.4% and 64.1%, respectively, Table 1). Correspondingly, the fertilization kinetics curves (Fig. 1) showed remarkable similarity between the treatments and replicates. The sperm concentration at which maximum fertilization was observed, S_{max}, did not differ markedly with pH (10864 and 10941 sperm µl^{-1} in pH 8.15 and pH 7.8, respectively, Table 1). These

differences were not statistically significant (ANOVA FSR_{max}, $F_{1,12}$=0.014, P=0.909; ANOVA S_{max}, $F_{1,12}$=0.631, P=0.442). Power analysis showed that these tests had >80% power to detect a change of 4.5% in maximum fertilization success (FSR_{max}).

4 Discussion

The absence of significant overall effects of pH on sperm swimming behaviour and fertilization success is remarkable (Fig. 1, Tables 1 and 2). Power analyses showed clearly that these results were not due to inadequate statistical power: our analyses had a high probability of detecting quite subtle effects of pH (5% change in response) – levels far lower than those reported for other species (Kurihara and Shirayama, 2004; Havenhand et al., 2008). This is an important result that allows us to conclude that the absence of significant effect is likely a true reflection of the responses of *Crassostrea gigas* gametes and zygotes from the Swedish west coast to levels of CO_2-induced acidification expected by the end of this century (Caldeira and Wickett, 2003; Blackford and Gilbert, 2007), rather than a result of low statistical power caused by excessive variation or inadequate replication.

Kurihara et al. (2007) also found no effect of CO_2-induced acidification (to pH 7.4) on fertilization in *C. gigas* from Japan. Those authors did not include a power analysis of their data, and it is not possible to reconstruct this from the data they provide. The variance in their data was, however, substantial (their Fig. 1) indicating that they may have lacked sufficient power to detect a subtle effects of pH at this stage of the life-cycle. Consequently it is difficult to place their results in context with the work presented here.

Other work by Kurihara's group has shown that after just 24 h, the larval stages of *C. gigas* are negatively impacted by CO_2-induced acidification at pH levels equivalent to those used here (Kurihara, 2008). This suggests that although fertilization success and early embryogenesis in this species are unlikely to be impacted by near-future levels of ocean acidification, actively calcifying larvae are more susceptible to such decreases in pH.

We observed variation in the response of sperm swimming behaviour to acidification that was statistically significant in 7 of 16 males (Table 1). It is tempting to suggest that this could be evidence for variable adaptive capacity of male *C.gigas* to respond to ocean acidification. If so, we would predict that the observed changes in sperm swimming behaviour would be reflected in the fertilization success of those males (i.e. males whose sperm swam faster at low pH should have correspondingly higher fertilization success and *vice versa*). At the sperm concentrations we used to assess motility (10^3–10^4 sperm μl^{-1}), proportional changes in swimming behaviour translate almost directly in the fertilization kinetics model (Styan, 1998) to effects on fertilization success (i.e. a 20% increase in swimming speed generates a ~20% increase in predicted fertilization success). This should be reflected in a significant positive linear relationship between changes in sperm swimming speed (Δspeed, Table 1) and changes in FSR_{max} (ΔFSR_{max}, Table 2). The relationship between these two variables was in fact non-significant and negative ($\Delta FSR_{max} = -0.18\Delta speed - 0.88$, $n=13$, $r^2=0.112$, $P=0.264$). Therefore it seems likely that the variability in responses of *C. gigas* sperm swimming to pH seen here do not translate into measurable differences in fertilization success. This result contrasts with the only comparable available data for sea urchins, where significant CO_2-induced reductions in sperm swimming behaviour were reflected in similar reductions in fertilization (Havenhand et al., 2008).

To our knowledge this is the first report of repeated assessment of the impacts of ocean acidification on a species from different ocean basins. This is an important step, and further extension to the geographic spread of sampling locations will greatly enhance our ability to extrapolate results from one location into a global context. Similarly, if we are to understand the and predict the full consequences of OA on marine organisms, our experiments need sufficient statistical power to detect biologically meaningful effects – i.e. maximise the likelihood that non-significant results are a reflection of no biological effect, and minimise the likelihood that such results are caused by insufficient replication for the levels of variation present in the experimental system. In this context, confirming the likely absence of significant impact of OA on a given species and

process is every bit as important as finding significant effects (negative or positive).

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Table 1. Sperm swimming behaviour in FSW and acidified FSW (acFSW). Speed=speed of motile sperm (i.e. >15 µm s^{-1}); $P(T \leq t)$ two tail, $P \leq 0.05$ marked in bold italics. Separate dates signify different single males. Grey text indicates experiments where only motility data are available, black text indicates experiments with motility *and* fertilization data (cf. Table 2).

Date	Trial	Salinity	pH FSW	pH acFSW	ΔpH	Speed FSW	Speed acFSW	ΔSpeed	*P*	% motile FSW	% motile acFSW	ΔMotile	*P*
30 Jul 2008		25.2	8.16	7.81	−0.35	102.3	98.2	−4.08	0.266	77.8	79.2	1.38	0.146
31 Jul 2008		21.4	8.16	7.81	−0.35	107.5	117.6	10.19	<0.001	71.8	69.4	−2.32	***0.030***
04 Aug 2008	E	22.4	8.21	7.81	−0.39	78.8	65.7	-13.13	***0.004***	62.0	56.6	−5.33	0.314
05 Aug 2008	F	22.5	8.16	7.83	−0.33	61.7	62.8	1.12	0.621	30.4	27.0	−3.44	0.397
06 Aug 2008	G	24.7	8.13	7.85	−0.28	82.1	69.7	-12.42	***0.027***	59.9	48.6	−11.33	0.498
07 Aug 2008		22.2	8.14	7.82	−0.32	57.8	67.9	10.17	0.135	28.1	29.3	1.20	0.115
11 Aug 2008	H	23.7	8.16	7.81	−0.35	81.8	78.7	-3.09	0.547	43.9	40.9	−3.02	0.269
13 Aug 2008	I	25.9	8.13	7.82	−0.31	72.6	81.1	8.50	***0.043***	48.8	56.6	7.81	0.911
14 Aug 2008	J	26.1	8.08	7.86	−0.22	102.7	111.7	9.00	***0.001***	46.9	50.4	3.58	0.494
15 Aug 2008	K	25.8	8.09	7.81	−0.28	103.4	125.8	22.38	<0.001	57.1	69.1	11.98	0.258
16 Aug 2008	L	25.9	8.09	7.81	−0.28	113.3	122.9	9.63	***0.025***	60.7	60.0	−0.74	0.598
18 Aug 2008	M	26.7	8.16	7.84	−0.32	82.5	83.9	1.39	0.690	59.1	52.2	−6.89	0.774
19 Aug 2008	N	22.1	8.12	7.82	−0.30	100.8	104.2	3.44	0.490	65.1	72.9	7.80	***0.017***
20 Aug 2008	O	23.9	8.18	7.80	−0.38	94.0	97.6	3.59	0.530	36.4	38.9	2.44	0.511
21 Aug 2008	P	24.1	8.18	7.80	−0.38	126.7	124.2	-2.46	0.815	69.2	65.4	−3.78	0.169
22 Aug 2008	Q	24.0	8.24	7.81	−0.43	105.8	97.5	-8.27	0.130	67.0	61.4	−5.61	0.257
Mean		24.20	8.15	7.82	−0.33	92.10	94.30	2.25		55.30	54.90	−0.39	
s.e.		0.42	0.01	0.00	0.01	4.77	5.52	2.35		3.70	3.80	1.53	

BGD
6, 4573–4586, 2009

Table 2. Fertilization success in FSW and acidified FSW (acFSW). FSR_{max}=maximum Fertilization Success Rate. S_{max} indicates sperm concentration that yielded FSR_{max}. Separate dates signify different single males. Grey text indicates experiments where only fertilization data are available, black text indicates experiments with motility *and* fertilization data (cf. Table 1).

Date	Trial	pH FSW	pH acFSW	ΔpH	# females	FSR_{max} FSW	FSR_{max} acFSW	ΔFSR_{max}	S_{max} FSW (sperm μl^{-1})	S_{max} acFSW (sperm μl^{-1})
21 Jul 2008	A	8.19	7.78	−0.41	3	86.0	84.0	2.0	7703	7703
22 Jul 2008	B	8.19	7.78	−0.41	4	82.0	88.0	−6.0	1388	1388
23 Jul 2008	C	8.13	7.85	−0.28	5	89.0	91.0	−2.0	9904	1650
24 Jul 2008	D	8.14	7.82	−0.32	3	92.0	88.0	4.0	11 666	1944
04 Aug 2008	E	8.21	7.81	−0.39	4	71.0	60.0	11.0	3,500	5,833
05 Aug 2008	F	8.16	7.83	−0.33	4	55.0	53.0	2.0	6,222	6,222
06 Aug 2008	G	8.13	7.85	−0.28	3	56.0	59.0	−3.0	30 000	30 000
11 Aug 2008	H	8.16	7.81	−0.35	3	37.0	46.0	−9.0	10 095	10 095
13 Aug 2008	I	8.13	7.82	−0.31	5	29.0	34.0	−5.0	6,933	6,933
14 Aug 2008	J	8.08	7.86	−0.22	3	60.0	59.0	1.0	33 333	33 333
15 Aug 2008	K	8.09	7.81	−0.28	3	58.0	66.0	−8.0	9,583	9,583
16 Aug 2008	L	8.09	7.81	−0.28	3	63.0	60.0	3.0	11 555	11 555
18 Aug 2008	M	8.16	7.84	−0.32	3	59.0	60.0	−1.0	20 250	20 250
19 Aug 2008	N	8.12	7.82	−0.30	3	65.0	64.0	1.0	3,458	3,458
20 Aug 2008	O	8.18	7.80	−0.38	3	57.0	60.0	−3.0	4,857	4,857
21 Aug 2008	P	8.18	7.80	−0.38	3	55.0	54.0	1.0	3,375	20 250
22 Aug 2008	Q	8.24	7.81	−0.43	1	71.0	76.0	−5.0	11 750	11 750
Mean		8.15	7.82	−0.33		63.38	64.13	−0.75	10 864	10 941
s.e.		0.01	0.01	0.01		4.36	4.00	1.25	2329	2473

BGD
6, 4573–4586, 2009

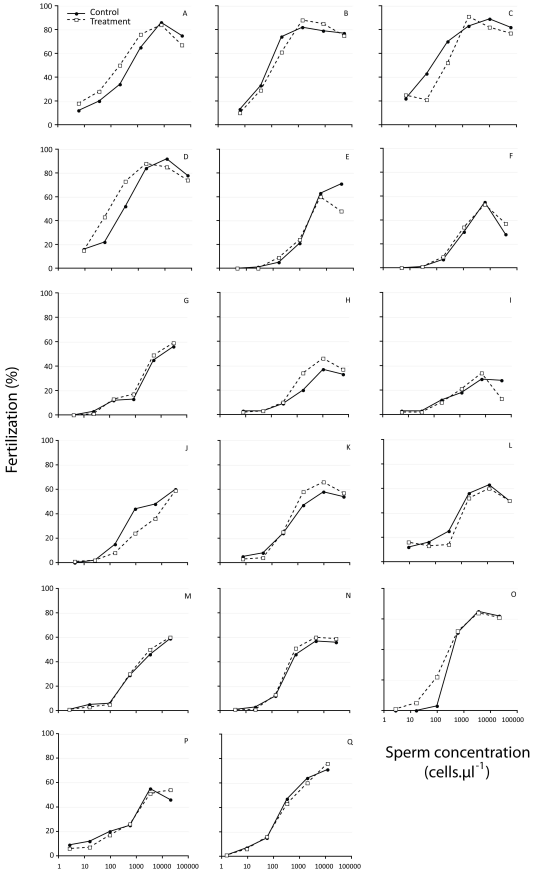

Fig. 1. Fertilization success and sperm concentration in "Control" (pH 8.15) and "Treatment" (pH 7.8) water. See Tables 1, 2 for key to males/dates and pH conditions.

BGD
6, 4573–4586, 2009