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RESEARCH ARTICLE

Ocean acidification impacts on sperm mitochondrial membrane potential bring sperm swimming behaviour near its tipping point

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ABSTRACT

Broadcast spawning marine invertebrates are susceptible to environmental stressors such as climate change, as their reproduction depends on the successful meeting and fertilization of gametes in the water column. Under near-future scenarios of ocean acidification, the swimming behaviour of marine invertebrate sperm is altered. We tested whether this was due to changes in sperm mitochondrial activity by investigating the effects of ocean acidification on sperm metabolism and swimming behaviour in the sea urchin *Centrostephanus rodgersii*. We used a fluorescent molecular probe (JC-1) and flow cytometry to visualize mitochondrial activity (measured as change in mitochondrial membrane potential, MMP). Sperm MMP was significantly reduced in ΔpH −0.3 (35% reduction) and ΔpH −0.5 (48% reduction) treatments, whereas sperm swimming behaviour was less sensitive with only slight changes (up to 11% decrease) observed overall. There was significant inter-individual variability in responses of sperm swimming behaviour and MMP to acidified seawater. We suggest it is likely that sperm exposed to these changes in pH are close to their tipping point in terms of physiological tolerance to acidity. Importantly, substantial inter-individual variation in responses of sperm swimming to ocean acidification may increase the scope for selection of resilient phenotypes, which, if heritable, could provide a basis for adaptation to future ocean acidification.

KEY WORDS: CO_2, Reproduction, Fertilization kinetics, Resilience, Sperm metabolism

INTRODUCTION

Sperm swimming behaviour is a key determinant of fertilization success (Fitzpatrick et al., 2012; Jantzen et al., 2001; Styan and Butler, 2000; Vogel et al., 1982). Ocean acidification impacts on sperm swimming behaviour have been investigated for a wide range of broadcast spawning marine invertebrates including oysters (Havenhand and Schlegel, 2009), sea urchins (Caldwell et al., 2011; Havenhand et al., 2008; Schlegel et al., 2012), corals (Morita et al., 2010; Nakamura and Morita, 2012), polychaetes (Lewis et al., 2013; Schlegel et al., 2014) and fish (Frommel et al., 2010). Most studies have reported reduced percentage motility or slower swimming speeds (or a combination of the two) under acidified conditions, while others have found no or slightly stimulatory impacts of ocean acidification on sperm swimming behaviour (Caldwell et al., 2011; Havenhand and Schlegel, 2009). Reduced sperm performance under acidified seawater conditions has been proposed to be caused by the effects of reduced pH on sperm metabolism (Lewis et al., 2013; Morita et al., 2010).

In sea urchins, sperm are stored immotile inside the testes in a CO_2-rich, acidic environment that inhibits respiration and motility (Darszon et al., 1999; Johnson et al., 1983). Once sperm are released into seawater, an uptake of sodium (Na^+) into the sperm cell triggers a release of hydrogen (H^+) ions (Bibring et al., 1984; Lee et al., 1983), causing a corresponding increase in internal pH (pH_i). This increased pH_i activates dynein-ATPase, which initiates mitochondrial activity and subsequent sperm motility (Christen et al., 1982, 1983; Hamamah and Gatti, 1998). Sperm energy metabolism is driven by pH-dependent mitochondrial respiration, which is reflected by the mitochondrial membrane potential (MMP) (Gibbons and Gibbons, 1972; Mita and Nakamura, 1998; Ruiz-Pesini et al., 2007). Correspondingly, sperm activity and MMP are correlated, with active sperm displaying higher MMP (Binet et al., 2014; Chen, 1988; Paoli et al., 2011; Schackmann et al., 1984). pH-dependent changes in MMP could thus be causal to observed reductions in sperm swimming behaviour under ocean acidification.

We investigated the relationship between pH, sperm swimming behaviour and mitochondrial membrane potential in multiple replicate individuals of the Australian sea urchin *Centrostephanus rodgersii* (A. Agassiz 1863) under simulated near- and far-future ocean acidification, based on Representative Concentration Pathway (RCP) and Special Report on Emissions Scenario (SRES) predictions: ΔpH=−0.3 units, RCP 8.5, with radiative forcing of 8.5 W m^{-2} for the year 2100 (Bopp et al., 2013); and ΔpH=−0.5 units, SRES 'A1FI' (fossil energy intensive scenario, with radiative forcing of approximately 9.1 W m^{-2}) for the year 2300 (Hofmann and Schellnhuber, 2009). *Centrostephanus rodgersii* is a major ecosystem engineer, transforming dense algal forests into barrens through intense grazing with ecosystem-wide effects on community composition and structure (Andrew, 1993; Andrew and Underwood, 1989). We combined flow cytometry with computerized sperm motility analyses to test the hypotheses that (i) ocean acidification will decrease MMP in sperm and reduce the proportion of motile sperm and sperm swimming speeds, and (ii) the magnitude of responses of sperm parameters to ocean acidification will vary significantly among individuals. Such variation in sperm swimming responses to acidified conditions could facilitate significant selection for sperm swimming behaviour.

RESULTS

Effects of collection date

Sperm were collected from males on two dates, 1 week apart (see Materials and methods); those in the first collection had slightly higher mean mitochondrial activity, percentage motility and sperm swimming speed than those in the second collection (supplementary material Table S1). We included the factor 'date' in our analyses

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(Table 1) in order to remove variance attributable to this variable. As our hypotheses addressed differences in response within and among males (nested within date), we do not consider the effects of 'date' further.

Effects of JC-1 stain

JC-1 staining had no effect on percentage motility and caused only small, non-significant, reductions in sperm swimming speed at different pH values (Fig. 1; ANOVA, pH×stain×male(date) P=0.883 and P=0.286 for percentage motility and sperm speed, respectively, Table 1A). There were, however, significant differences among the responses of different males to the stain (ANOVA, male×stain, P<0.001 for both sperm speed and percentage motility; Table 1A). Across all three pH treatments, JC-1 had a mean effect of −0.4% on percentage motility (ranging from +5.5% to −6.1% between individuals) and −4.7% on sperm speed (ranging from +8.5% to −16% between individuals). Consequently, for all further comparisons between sperm performance and MMP, we used data for sperm incubated with JC-1. Importantly, however, staining with JC-1 had no significant impact on sperm swimming responses at different pH (ANOVA, pH×stain P=0.890 and P=0.113 for percentage motility and sperm speed, respectively; Table 1A), indicating that despite among-male differences in staining, we could still compare responses to the different treatments within replicate males.

MMP (FL2/FL1 ratio)

Decreasing pH caused a significant decrease in MMP for sperm from all males (Figs 2 and 3). The 95% confidence intervals (CI) around mean individual responses (ln response ratios; Fig. 2) showed a reduction in MMP of −0.30 to −0.54 (equivalent to −26% to −42%, with a mean of −35%) at ΔpH −0.3 and a reduction in MMP of −0.50 to −0.80 (equivalent to −39% to −55%, and a mean of −48%) at ΔpH −0.5.

Proportion of motile sperm

The effects of ocean acidification on percentage sperm motility differed significantly among males [ANOVA, pH×male(date) P<0.001, Table 1A]. Some males showed higher proportions of motile sperm under mildly acidified conditions when compared with present day conditions, whereas this pattern was reversed at lower pH (*post hoc* Tukey's, P<0.001; Figs 2 and 3). In all cases, biological effect sizes were relatively small (Fig. 2): responses of single males to ΔpH −0.3 varied from a moderate increase to a slight decrease in percentage motility (upper and lower bound ln response ratio 95% CI of +0.084 to −0.010, equivalent to +8.8% to −1.0% in stained sperm, mean +3.4%); at ΔpH −0.5, variation in responses ranged from slight increases to moderate decreases in percentage motility (ln response ratio 95% CI were +0.0092 to −0.11, equivalent to +0.92% to −11% in stained sperm, mean −4.8%).

Sperm swimming speed

Responses of sperm swimming speed to ocean acidification followed similar patterns to those of percentage motility. Here again, effects of pH differed significantly among males [ANOVA, pH×male(date) P<0.001; Table 1A]. Sperm swimming speed was fastest at ΔpH −0.3 and slowest at ΔpH −0.5 (*post hoc* Tukey's, P<0.001; Fig. 2). The 95% CI for individual responses (ln response ratios) were +0.17 to −0.043, equivalent to changes in sperm speed of +18% to −4.2% (mean +5.8%), and −0.021 to −0.21, equivalent to changes in sperm speed of −2.0% to −19% (mean −11%) for ΔpH −0.3 and ΔpH −0.5, respectively.

Table 1. Effect of stain, male, pH and mitochondrial membrane potential (MMP) on the proportion of motile sperm and sperm swimming speed

	% Motility				Speed of motile sperm			
A	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
pH	2	0.146	4.83	**0.023**	2	6598	7.28	**0.006**
Stain	1	0.011	0.28	0.609	1	3799	2.49	0.153
Date	1	8.804	1702		1	165,715	1366	
Male(date)	7	0.258	49.81	**<0.001**	7	9732	80.2	**<0.001**
pH×stain	2	0.000	0.118	0.890	2	357	2.51	0.113
pH×male(date)	16	0.030	9.73	**<0.001**	16	906	6.37	**<0.001**
Stain×male(date)	8	0.039	12.4	**<0.001**	8	1525	10.7	**<0.001**
pH×stain×male(date)	16	0.003	0.601	0.883	16	142	1.17	0.286
Residual	432	0.005			432	121		
	% Motility×FL2/FL1 ratio				Speed×FL2/FL1 ratio			
B	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Fixed factors								
pH	2	0.0268	5.76	**0.004**	2	664.4	6.800	**<0.001**
FL2/FL1 ratio	1	0.0109	2.34	0.128	1	89.5	0.916	0.346
pH×FL2/FL1 ratio	2	0.018	3.86	**0.023**	2	147.4	1.508	0.195
		d.f.	Variance			d.f.	Variance	
Random factors								
Date			468.8				0.0301	
Male(date)			132.5				0.0048	
pH×male(date)			25.1				0.00091	
Residual		213	97.7			213	0.0046	

(A) Multifactor ANOVA results for the impact of JC-1 stain, pH and male on the proportion of motile sperm and sperm swimming speed [pH and stain=fixed, date and male (nested within date)=random]. (B) Linear mixed model ANCOVA to test for effects of mitochondrial activity (MMP; measured as FL2/FL1 ratio), pH and male on the proportion of motile sperm and sperm swimming speed [pH and MMP=fixed, date and male (nested within date)=random].
Significant effects (P≤0.05) are shown in bold, significance values for random terms in B were calculated by χ^2 tests of full versus reduced models in package *lme4* within the R environment.

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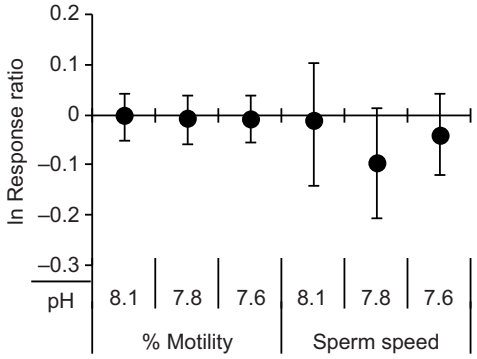

Fig. 1. Effects of JC-1 staining on sperm swimming behaviour of *Centrostephanus rodgersii* at different pH levels. Response ratios are the ln response of stained sperm divided by the response of unstained sperm. Data are bootstrapped means ±95% confidence intervals (CI). Larger CI indicate greater between-male variability in responses. Values below zero indicate inhibitory effects of the stain on sperm parameters. *N*=9.

Relationship between sperm swimming behaviour and MMP

Reductions in MMP in acidified treatments had varying relationships with the different sperm swimming parameters (Fig. 3). ANCOVA on sperm percentage motility data showed significant covariance with MMP, but the strength of this relationship was different among the treatments (Fig. 3A; ANCOVA, pH×MMP P=0.023; Table 1B). Analysis of individual relationships for each pH indicated that this pattern was driven primarily by responses in acidified treatments (significant correlations between percentage motility and MMP were only observed in the ΔpH −0.3 and ΔpH −0.5 treatments, Fig. 3A). Similar patterns were observed for the relationships between MMP and sperm swimming speed at different pH (Fig. 3B), although in this case there were no statistically significant interactions between the MMP–speed relationships and pH (ANCOVA, pH×MMP P=0.195, Table 1B).

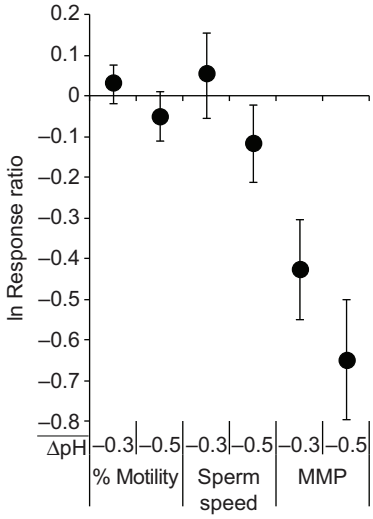

Fig. 2. Effects of ocean acidification (ΔpH) on three sperm performance metrics in *C. rodgersii*. Percentage sperm motility, sperm speed and mitochondrial membrane potential (MMP) data are bootstrapped mean individual response ratios (ln of response under acidified conditions divided by response under ambient conditions) ±95% CI. Larger CI indicate greater between-male variability in responses. Values below zero indicate inhibitory effects of ocean acidification on sperm parameters. *N*=9.

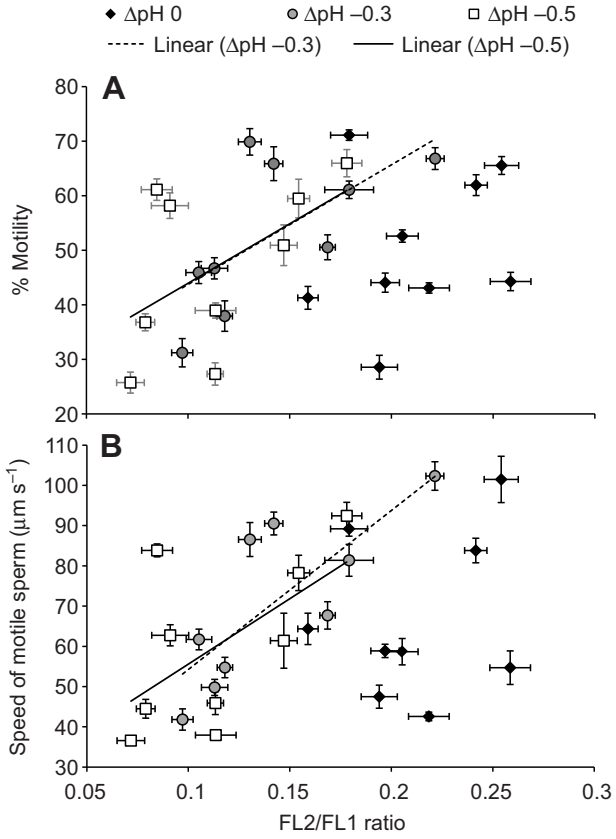

Fig. 3. Relationship between MMP (measured as FL2/FL1 ratio) and sperm swimming behaviour in JC-1-stained sperm of nine male *C. rodgersii* at different pH levels. (A) Proportion of motile sperm and (B) swimming speed of motile sperm. Means±s.e., *N*=9. Percentage motility, r^2=0.02, 0.24***, 0.20***; sperm speed, r^2=0.01, 0.41***, 0.22*** (calculated from raw data for pH 8.1, 7.8, 7.6, respectively, *N*=81, asterisks indicate statistical significance ***P≤0.001). Trend lines have been added where significant relationships were found.

DISCUSSION

Sperm swimming behaviour and MMP of *C. rodgersii* were significantly affected by ocean acidification. Sperm MMP was reduced under both near-future (ΔpH −0.3) and far-future (ΔpH −0.5) ocean acidification scenarios (Fig. 2). These responses were not, however, fully reflected in sperm performance. In acidified treatments, we found strong relationships between sperm swimming speed and MMP, and sperm percentage motility and MMP, but these relationships were not present for sperm in ambient (pH 8.1) seawater (Fig. 3).

In activated sea urchin sperm, the major source of energy (ATP) required for motility is mitochondrial respiration, with some species also deriving additional ATP from glyolysis (Christen et al., 1982; Mita and Nakamura, 1998). MMP reflects mitochondrial respiratory activity, such that active sperm typically display higher MMP than inactive sperm (Binet et al., 2014; Chen, 1988; Paoli et al., 2011; Schackmann et al., 1984). Parallel studies with *C. rodgersii* show that sperm swimming behaviour (motility and speed) is reduced under acidified conditions (P.S., J.N.H., S. Milfsud, O. Hoegh-Guldberg and J.E.W., unpublished). The significant relationship between decreased MMP and decreased speed/motility with increased acidity was therefore expected, and indicates that reduced sperm swimming under ocean acidification is probably due to reduced mitochondrial activity in those sperm. In ambient

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seawater (pH 8.1), however, there was no clear relationship between MMP and sperm speed/motility. This result arose because sperm from a small number of individuals, when activated in ambient (pH 8.1) seawater, had elevated MMP but low motility/speed (Fig. 3). The reasons for this are not known, although it is possible that the mechanism that determines the MMP–motility relationship is saturated at high pH.

Saturation of the MMP–motility relationship at pH 8.1 could arise through this species being at the 'tipping point' of tolerance to pH change. Tolerance of a species to environmental stressors is typically determined from dose–response curves, which often follow a monotonic, sigmoidal relationship (Scholze et al., 2001). Typically, organisms tolerate the stressor up to a tipping point, after which there is a steep drop in performance, ultimately leading to complete inhibition of the response variable. Our chosen experimental design prioritized levels of replication measurements (N=9) and males (N=9), at the cost of the number of treatment levels (N=3: control, ΔpH −0.3, ΔpH −0.5). This lack of resolution in the effect of treatment (i.e. acidification) inevitably limited our ability to model the nature of the relationships between pH, MMP and swimming behaviour. Furthermore, tolerance curves often experience hormesis effects at the top of the sigmoidal curve, where slight amounts of the stressor can actually enhance organism response (Calabrese, 2008). This may explain the effects we observed where motility was slightly increased at ΔpH −0.3. In this case, the enhanced motility response may have been caused by stimulated enzyme activity. There is evidence to suggest that sea urchins from different phylogenetic groups utilize different endogenous sources for oxidation, and that enzymes regulating this process have different optimal pH. For example, lipase activity in *Glyptocidaris crenularis* (order Arbacioda) peaks at pH 7.5, whereas phospholipase A_2 activity in *Hemicentrotus pulcherrimus* (order Echinoida) peaks at pH 8.0 (Mita and Nakamura, 1998). When pH is lower or higher than these peak values, enzyme activity is decreased. While the specific oxidation mechanisms in *C. rodgersii* (order Diadematoida) are not known, it is possible that at ΔpH −0.3 (pH 7.8), enzyme activity was stimulated, thereby increasing ATP sufficiently so as to also increase sperm motility. At ΔpH −0.5, enzyme activity was probably reduced, so that in addition to reduced MMP, a reduction in oxidation may have contributed to the observed decrease in motility and swimming speed. Future studies that include more treatment levels would permit more accurate modelling of the relationships between pH, swimming behaviour and MMP through the use of point-estimate or Bayesian models similar to those typically used in ecotoxicology (van der Hoeven, 2004, 1997; Fox, 2010). In addition, measurement of oxidation/glycolysis and enzyme activity could provide a more complete understanding of the effects of ocean acidification on sperm motility.

The possibility that peak motility/sperm swimming speed did not occur at the time that our measurements were taken may also have limited our ability to draw clear conclusions regarding the observed patterns. Under present day conditions (seawater pH 8.1), activation of sperm from marine broadcast spawners is believed to occur almost instantly upon release into seawater (Christen et al., 1982, 1983). Contact with seawater causes an influx of external Na^+ into the sperm that triggers the release of excess H^+ ions from within the cell, elevating pH_i. The rise in pH_i triggers the activity of dynein ATPase, which in turn initiates respiration in the sperm (Bibring et al., 1984; Christen et al., 1983; Lee et al., 1983). After activation, sea urchin sperm are reliant on endogenous sources for their energy metabolism (Mita and Nakamura, 1998), and activity levels decrease over time as energy resources become depleted (Levitan, 2000). Increased external H^+ concentrations at lowered external pH (pH_e; as predicted under future ocean acidification) will weaken the gradient between pH_e and pH_i in sea urchin sperm, thus decreasing pH_i, so increasing sperm activation time and reducing/delaying their maximum motility (Yu et al., 2011). We measured sperm motility 15 min after activation of sperm in seawater treatments to allow for efficient internalization of JC-1 into the mitochondria, and because it was previously shown that *C. rodgersii* sperm motility after 20 min incubation at 18°C was similar to that measured immediately following activation (Binet et al., 2014). We suggest that increased temporal resolution in sperm motility measurements in future studies, rather than one-off snapshots of motility, will permit determination of the timing of sperm activation and peak motility, and hence whether they are in fact delayed under acidified conditions.

Lowered mitochondrial activity under ocean acidification, and hence lowered consumption of the limited endogenous energy reserves (Mita and Nakamura, 1998), should also increase sperm longevity – something that was not measured here. Increased sperm longevity increases fertilization success when sperm–egg encounter rates remain high over prolonged periods (Vogel et al., 1982), for instance during synchronized spawning events (Fitzpatrick et al., 2012; Levitan, 2000; Marshall, 2002). So, while it has been shown that reduced sperm activity can explain observed reductions in fertilization success under ocean acidification (Havenhand et al., 2008), it is nonetheless possible that acidification-induced increases in sperm longevity could increase fertilization success over periods greater than the usual time frame measured in fertilization assays (≤30 min). Ultimately, however, fertilization involves both sperm and eggs, and research on the effects of acidification on the receptivity of the egg for sperm, chemotaxis, the fertilization process itself and the time course of sperm activity is lacking.

Patterns of sperm swimming behaviour in response to acidification that we observed here are consistent with recent findings in another species of sea urchin. Caldwell et al. (2011) reported an increased proportion of motile sperm and swimming speeds in *Psammechinus miliaris* under near-future conditions of ocean acidification (ΔpH −0.3), and slight decreases under far-future conditions (ΔpH −0.5). A similar study on *C. rodgersii*, however, found inhibitory effects of both near- and far-future ocean acidification (ΔpH −0.3 and −0.5, respectively) on sperm swimming behaviour (Schlegel et al., in review). These differences may have arisen because responses – and hence gamete quality – can vary with collection date (Table 1) and/or parental experience (Crean et al., 2013; Jensen et al., 2014). Differences in parental genotypes and environments can directly affect gamete plasticity and performance (Crean et al., 2013; Crean and Marshall, 2008; Jensen et al., 2014). A similar robustness to climate change has been found in the gene network of the urchin *Strongylocentrotus purpuratus* (Runcie et al., 2012): elevated seawater temperatures caused perturbations of regulatory genes, which were buffered effectively within the network and did not affect the expression of regulated genes. Ultimately, it should be stressed that while understanding the impacts of climate change on the underlying mechanisms is essential, it is the functional responses that drive ecology and evolution.

In conclusion, MMP and sperm swimming speed in *C. rodgersii* were reduced under ocean acidification. The decreases in overall MMP under ocean acidification did not translate directly into

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equivalent changes in sperm swimming behaviour. Analysis of the covariance between sperm swimming performance and MMP suggests that this relationship may be pH dependent; however, further work incorporating a full pH–response curve is needed to clarify this relationship. Whether the substantial inter-individual variation we observed in sperm responses to ocean acidification could provide a basis for the selection of resilient phenotypes and/or is the result of transgenerational plasticity remains to be determined. Further research identifying the respective contributions of plastic and genetic variation to acidification resistance at the gametic level will provide valuable insights into the nature and extent of pre-existing adaptive variations, and benefit our understanding of the capacity for acclimation and adaptation to near-future ocean acidification.

MATERIALS AND METHODS

Specimen and gamete collection

Centrostephanus rodgersii were collected under scientific collection licence P10/0021-1.2 from the Department of Primary Industries, NSW, Australia, towards the end of the spawning season in August 2012 (males 1–5 on 9 August and males 6–9 on 16 August) from shallow rocky subtidal areas at Fairlight, Sydney, Australia (33°48′1″S, 151°16′3″E). Animals were transported to Macquarie University in aerated containers, and then held in a 30,000 l recirculating seawater system until used in experiments (≤1 week). During this time, animals were fed *ad libitum* their preferred food, the fresh kelp *Ecklonia radiata*. Immediately prior to experiments, spawning was induced via a single non-lethal injection of 1–2 ml of 0.5 mol l^{-1} KCl through the peristomal membrane. Only individuals that spawned readily after injection were used in experiments. Released sperm were collected 'dry' (i.e. in an empty vial) on ice. Separate vials were used for sperm from each male.

Water treatment

Experimental pH_{NBS} (calibrated in dilute National Bureau of Standards buffers) was adjusted by bubbling a mixture of filtered air and CO_2 through filtered seawater (FSW; 0.22 µm). Microprocessor-controlled CO_2 injection units were used to maintain pH changes (ΔpH) of 0 (no CO_2 addition; analogous to current conditions), −0.3 and −0.5 pH units (analogous to projected conditions in coastal waters in 2100 and in 2300, respectively, under the RCP8.5 and A1FI scenarios; Bopp et al., 2013; Hofmann and Schellnhuber, 2009) in separate FSW tanks. Resultant pH levels were 8.1 (control; partial CO_2 pressure P_{CO_2}=435 µatm), 7.8 (P_{CO_2}=950 µatm) and 7.6 (P_{CO_2}=1558 µatm). Seawater temperature and salinity were measured directly for each replicate (N=9) using an IQ Sensor net (MIQ/T2020, WTW, Weilheim, Germany). Total alkalinity was determined for every third replicate (N=3) by titration (HI 3811 Alkalinity, Hanna Instruments, Woonsocket, RI, USA). Carbonate system parameters were calculated using CO2-SYS (Lewis and Wallace, 1998) using the dissociation constants of Dickson and Millero (1987) (Table 2).

Preparation of sperm suspensions and exposures

The experiment was carried out a total of nine times, using sperm from a different male for each experiment (i.e. total of N=9 males,). Within each experiment, sperm from one male was extracted and stored 'dry' (undiluted) on ice. Nine new 3.5 µl aliquots of the dry sperm were directly activated in each pH treatment solution, forming nine replicate exposures and measurements per treatment, per male. Resultant sperm suspensions in each treatment [2–4 (×10^4) sperm $µl^{-1}$) were immediately divided into three 500 µl aliquots. Mitochondrial stain 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrathylbenzimidazolyl-carbocyanine iodide (JC-1; Sigma-Aldrich, St Louis, MO, USA) was added to two of the three aliquots (final concentration 0.25 µmol l^{-1}; for details on JC-1 stock preparation, see Binet et al., 2014). All three aliquots were incubated in the dark for 15 min (T=18±1°C) to ensure effective staining and control for these effects. After this incubation time, sperm swimming behaviour (motility and speed) was recorded for one JC-1-stained and one unstained aliquot, while flow cytometry was used to assess mitochondrial membrane potential in sperm from final JC-1-stained aliquots.

Table 2. Seawater parameters

pH_{NBS}	T (°C)	Salinity	A_T (µequiv kg^{-1})	P_{CO2} (µatm)	Ω_{Ca}	Ω_{Ar}
8.10±0.01	18±1	35.5±0.1	2106±4	435	3.4	2.2
7.80±0.01	18±1	35.5±0.1	2106±4	950	1.8	1.2
7.60±0.01	18±1	35.5±0.1	2106±4	1558	1.2	0.8

Direct measurements taken for pH_{NBS} (calibrated in dilute National Bureau of Standards buffers), temperature (T), salinity and total alkalinity (A_T) were used to calculate partial CO_2 pressure (P_{CO2}) and seawater saturation states for calcite (Ω_{Ca}) and aragonite (Ω_{Ar}) using CO2-SYS (see Materials and methods). Data are means±s.e., N=9 for pH, T and salinity; N=3 for A_T.

Flow cytometry

Flow cytometry measurements were performed following a protocol for *C. rodgersii* developed by Binet et al. (2014): JC-1 stained sperm were analysed using a Cell Lab Quanta SC-MPL (Beckman Coulter, Brea, CA, USA) flow cytometer, equipped with a blue laser (488 nm). Channels measured fluorescence at two wavelengths: green FL1 (525/30 nm) and orange FL2 (575/30 nm). JC-1 has a green-emitting, monomeric form at low MMP, but forms orange/green-emitting aggregates at high MMP. Data were post-processed using Cell Lab Quanta SC-MPL software and plotted on cytograms of FL1 versus FL2 (supplementary material Fig. S1). The ratio of FL2/FL1 for JC-1 is generally used to measure MMP in stained cells (Cossarizza, 1997; Cossarizza and Salvioli, 2000), and allows for differentiation of cells with high or weak MMP. Here, healthy sperm with high MMP fluoresced brightly in green (FL1) and in orange (FL2), and fell into the top-right quadrant of the cytogram, resulting in high FL2/FL1 ratios (supplementary material Fig. S1B). Sperm with decreased MMP displayed lower orange (FL2) fluorescence and higher green (FL1) fluorescence, and moved into the lower-right quadrant, resulting in lower, and generally non-overlapping, FL2/FL1 ratios (supplementary material Fig. S1C).

Sperm motility

Experiments followed established protocols (Havenhand et al., 2008; Havenhand and Schlegel, 2009; Schlegel et al., 2012). Briefly, for each replicate (N=9, for each male) and male (N=9), a small drop of sperm suspension (~60 µl) was placed on an albumin-coated microscope slide and coverslip, separated by a 0.75 mm thick O-ring. Sperm movement under DIC illumination was recorded at the midpoint of the drop in the O-ring for 2 s at 50 frames s^{-1}, using a digital camera (Sumix SMX-160, Oceanside, CA, USA) mounted on a compound microscope (Olympus BX53, Tokyo, Japan). Videos were post-processed and analysed using CellTrak 1.3 (Motion Analysis Corporation, Santa Rosa, CA, USA). The proportion of motile sperm (defined as sperm swimming faster than 20 µm s^{-1}) and their swimming speed were recorded for each slide.

Statistical analyses

All percentage data were arc-sin transformed prior to statistical analyses. Box plots and Levene's test were used to assess data for normality and homogeneity of variance (Quinn and Keough, 2002). The statistical significance of effects of pH, JC-1 stain, male and collection date on sperm performance (proportion of motile sperm and swimming speed) were determined using ANOVA in SPSS™. Differences between means were compared *post hoc* using Tukey's test. The effects of pH, male and collection date on the covariance between mitochondrial membrane potential (measured as FL2/FL1 ratio) and sperm performance (speed or motility) were determined using linear mixed effects models in the R framework (R Development Core Team, 2012). Variation in responses of sperm parameters to pH among males was assessed using logarithmic response ratios (natural log of treatment response divided by control response; Hedges et al., 1999). 95% CI around mean ln response ratios were determined by bootstrapping in R (100,000 iterations).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors contributed to conception and design of the experiments. P.S. conducted experimental work, organized and conducted field collection of animals (including permits), and drafted the article. M.T.B. conducted experimental work, provided funding and equipment, and contributed to drafting and revision of the article. J.N.H. revised the article and contributed to theory formulation. C.J.D. revised the article and contributed to theory formulation. J.E.W. provided funding, organized equipment, facilitated animal collection and revised the article.

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