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Interactive effects of ocean warming and acidification on sperm motility and fertilization in the mussel *Mytilus galloprovincialis*

Angela R. Eads*, W. Jason Kennington, Jonathan P. Evans

Centre for Evolutionary Biology, University of Western Australia, Crawley, WA 6009, Australia

ABSTRACT: Gametes of marine broadcast spawners are highly susceptible to the threats of ocean warming and acidification. Here, we explore the main and interacting effects of temperature and pH changes on sperm motility and fertilization rates in the mussel *Mytilus galloprovincialis*. Additionally, we determine how temperature and pH interact to influence the motility of aging sperm. We show that the interactive effects of temperature (18°C or 24°C) and pH (ranging from 7.6 to 8.0) on sperm motility depend on the time that sperm spend in these conditions. Specifically, sperm linearity was influenced by a temperature × pH interaction when measured after a relatively short exposure to the treatment conditions, while main effects of temperature and pH (but no interaction) on sperm motility became apparent only after prolonged exposure (2 h) to the treatments. Despite the interactive effects of temperature and pH on sperm motility, these factors had independent effects on fertilization rates, which were significantly higher at the ambient ocean pH level and at the elevated temperature. This study highlights the importance of considering the combined effects of predicted ocean changes on sperm motility and fertilization rates, and cautions against using only sperm motility as a proxy for reproductive fitness. Detrimental effects of pH and temperature may only be uncovered when these factors are examined together, or conversely, negative impacts of one variable may be buffered by changes in another. Our results raise the intriguing possibility that some species may cope better with ocean acidification if they simultaneously experience ocean warming.

KEY WORDS: Ocean acidification · Ocean warming · Sperm motility · Fertilization · Broadcast spawner

INTRODUCTION

Many sessile marine invertebrates reproduce by broadcast spawning, where gametes are released directly into the water column for external fertilization. While fertilization is often considered the first hurdle for these organisms under stressful conditions (Byrne et al. 2010), gametes themselves face the challenge of locating each other for fertilization in a highly disruptive fluid environment. For successful external fertilization in dioecious broadcast spawners, sperm must first locate (e.g. through sperm chemotaxis) and then swim towards eggs from genetically compatible females, often in competition with sperm from rival males (reviewed by Evans & Sherman 2013).

In addition to the inherent challenges faced by sperm in locating eggs and competing for fertilizations, unprecedented changes to ocean conditions brought on by anthropogenic climate change will introduce gametes to a new suite of stressors (Raven et al. 2005, Doney et al. 2009, IPCC 2013). Of particular concern are the effects of increasing temperature and reduced pH on gamete survival and communication, as well as other reproductive life stages, such as fertilization, embryonic development, and larval growth (Kurihara 2008, Brierley & Kingsford

*Corresponding author: angela.eads@graduate.uwa.edu.au

2009, Byrne 2011, Byrne & Przeslawski 2013). However, there have been mixed results from studies that have explored how sperm behavior is influenced by changes in seawater temperature and/or pH. On the one hand, decreased pH has been shown to impair sperm motility (Nakamura & Morita 2012, Vihtakari et al. 2013, Schlegel et al. 2014), while on the other, a number of studies have revealed no effect or even positive effects of decreasing seawater pH on sperm velocity (Havenhand & Schlegel 2009, Schlegel et al. 2012, Lewis et al. 2013, Graham et al. 2016). Similarly, higher water temperature has been shown to have both negative and positive effects on sperm motility across a range of species, including Japanese eel (Huang et al. 2011), sticklebacks (Mehlis & Bakker 2014), and other fish species (Alavi & Cosson 2005, Purchase et al. 2010; see references therein for potential molecular, physiological, and behavioral mechanisms causing observed changes in sperm motility). Disruption to this very early reproductive stage, whether by complete failure to find and fertilize eggs or by losing the ability to detect compatible mates, may have serious impacts on fertilization rates and individual reproductive fitness, potentially leading to population extinctions (Byrne 2012).

In this study, we investigate the main and interacting effects of changes in seawater temperature and pH on sperm motility and fertilization rates in the Mediterranean mussel *Mytilus galloprovincialis*. In this way, we hope to gain an understanding of the capacity of mussel sperm to tolerate predicted concurrent changes in ocean temperature and pH. *Mytilus* spp. are cosmopolitan broadcast spawners that occupy marine intertidal habitats in temperate to subarctic waters of the Northern and Southern hemispheres. *Mytilus* spp. sperm swim in circular motions, which is likely to enable them to avoid swimming too far before detecting chemoattractants produced by eggs (Evans et al. 2012). It may, therefore, be effective for sperm to restrict their movement by swimming slowly and in tight circles to maximize their chances of encountering compatible eggs in a turbulent intertidal habitat (Liu et al. 2011, Fitzpatrick et al. 2012, Stewart et al. 2012). To evaluate the main and interacting effects of both environmental factors on a range of sperm motility parameters, we used a factorial experimental design in which we assessed the motility of each male's sperm across a range of 3 pH levels and 2 temperatures in all combinations. Furthermore, because sperm from some mussel species can remain motile and viable for up to 67 h (Sprung & Bayne 1984; *M. edulis*), we consider the effect of these environmental stressors over time to determine whether changes in ocean temperature and pH compound aging effects on sperm motility (Williams & Bentley 2002). Finally, the fertilizing ability of sperm under identical conditions was assessed as a fitness proxy for gametes in these environmental conditions.

MATERIALS AND METHODS

Study species, collection, and maintenance

The cosmopolitan broadcast spawner *Mytilus galloprovincialis* is considered an ecosystem engineer due to the formation of mussel beds that provide habitat for numerous organisms (Gutiérrez et al. 2003). The range of *M. galloprovincialis* in Australia is limited to the southern half of the country's coastline, most likely due to limits in thermal tolerance (Suchanek 1978, Dias et al. 2014). These habitats correspond with areas where the rate of ocean warming has been greatest, attributable to poleward-flowing currents (Pearce & Feng 2007, Lough et al. 2012). The temperature increases of the Leeuwin Current region are most pronounced in winter when the current is strongest (Caputi et al. 2009, Lough et al. 2012), corresponding with the mussel's spawning season in Australia (Wilson & Hodgkin 1967). Sea surface temperatures off the Australian coastal regions inhabited by *M. galloprovincialis* are predicted to rise up to 3.5°C over the coming century (Hobday & Lough 2011, Lough et al. 2012, IPCC 2013), while heatwaves off the coast of Western Australia in 2010 and 2011 saw temperatures rise by 5°C (Pearce & Feng 2013). Alongside the ease of collecting, maintaining, and spawning mussels, these environmental considerations make *M. galloprovincialis* an ideal test subject for our study.

Mussels were collected by hand from a pontoon at Woodman Point, 30 km south of Perth, Western Australia, over multiple trips from July to September during 2012 to 2014 (permit no. 2141, Department of Transport, Government of Western Australia). Experimental replicates were taken over multiple seasons due to poor spawning, and year was included in the analyses to account for any seasonal variation (see 'Statistical analyses'). Winter water conditions at the collection site averaged 19.8°C and pH 8.1 over the experimental seasons (Reynolds et al. 2007; https://imos.aodn.org.au/imos123). Mussels were kept in aerated aquaria of recirculating biologically filtered seawater (FSW) at the University of Western Australia until required (within 3 wk of collection).

Seawater preparation

Experimental water temperatures were maintained by placing containers in water baths in a temperature-controlled room. A total of 4 water baths, comprising 2 replicates of each experimental temperature, were used in the experiment. Holding containers with FSW were placed in each water bath and, after water temperatures were stable, experimental pH levels were set by bubbling CO_2 through the FSW. By using CO_2 to alter seawater pH, the partial pressure of CO_2 (pCO_2) is raised while simultaneously lowering the pH and carbonate ion concentrations. (Lowering the pH of water by adding an acid such as HCl maintains the pCO_2—unrealistic conditions in which to investigate the impacts of anthropogenic climate change—and prior research has shown altered outcomes on fertilization rates using this method; Sung et al. 2014).

Experimental water temperatures were 18°C and 24°C, while pH levels were set at 7.6, 7.8, and the local ambient pH (~8.0). These values represent current and predicted average winter conditions for the local area in the coming century and are therefore likely to be realistic for the focal population, particularly considering increases in marine heatwave occurrence and length along with more extreme peaks in environmental fluctuations (IPCC 2013, Pearce & Feng 2013). Water parameters (pH, temperature, dissolved oxygen, and salinity) were measured before and after conducting each experimental 'block' (= male) using a pH meter (TPS WP-81; TPS Pty Ltd) calibrated with TPS buffers, and $pCO2$ ranges were calculated using CO2SYS software (Pierrot et al. 2006; Table S1 in the Supplement at www.int-res.com/articles/suppl/m562p101_supp.pdf). For each 'block', we placed 3 × 150 ml glass jars, each containing 30 ml of water set at one of the 3 pH levels (~8.0, 7.8, or 7.6), in each water bath (18°C or 24°C).

Spawning and gamete collection

Mussels were induced to spawn using a temperature shock by moving them from their holding tanks (at ~17°C) to a large tray preheated to ~26°C using an aquarium heater (SONPAR automatic, 200W) (Galley et al. 2010). Females that began spawning were rinsed in FSW, placed in a glass jar containing 30 ml of ambient FSW, and left for approximately 1 h to spawn. When a male commenced spawning, it was removed from the tray, rinsed in FSW, and wrapped in a wet paper towel to halt spawning until enough eggs were collected (see below). When required, each male was placed in a glass spawning jar containing 30 ml of ambient FSW and left to spawn for approximately 10 min until sperm were sufficiently concentrated (as judged initially by eye) for the sperm motility and fertilization assays (see below). Sperm density in the spawning jar was quantified using an improved Neubauer haemocytometer (Hirschmann Laborgeräte). We then extracted a known quantity of sperm from the spawning jars to make up concentrations of 5×10^6 sperm per ml in each treatment jar, a concentration appropriate for the sperm motility analysis and fertilization trials below (see below).

Characterizing sperm motility

Sperm motility of a subset of males (n = 14) was assessed using computer-assisted sperm analysis (CASA; CEROS sperm tracker, Hamilton-Thorne Research), 20 min after sperm were added to the treatment water, and then again 2 h post-addition to treatment conditions (hereafter referred to as Time 1 and Time 2, respectively). For each sample, 1.5 µl of sperm was pipetted into 2 separate wells of a 12-well multitest slide (MP Biomedicals) and covered with a coverslip. We used a phase-contrast Olympus CX41 microscope (×10 objective) and captured 30 frames at 50 f s^{-1}. We defined static cells below the threshold values of 19.9 µm s^{-1} for smoothed average path velocity (VAP) and 4 µm s^{-1} for straight-line velocity (VSL) (see 'Statistical analyses' for details about the CASA parameters), minimum cell size as 2 pixels, and measured an average of 193 ± 8 SE sperm tracks per sample. The slides were coated with 1% polyvinyl alcohol (Sigma-Aldrich) to avoid sperm sticking to the glass (Wilson-Leedy & Ingermann 2007). We randomized the order in which sperm motility was analyzed by treatment among males.

Fertilization trials

In each water bath (18°C or 24°C), 3 × 50 ml plastic tubes were floated in a polystyrene frame, each containing 10 ml of water set at one of the 3 pH levels (~8.0, 7.8, or 7.6). Eggs from all females spawned on a given day were pooled (range: 2–6 females) to provide a 'homogenous' genetic background for the fertilization trials (Fitzpatrick et al. 2012), thus reducing variance in fertilization rates attributable to specific male-by-female interactions (i.e. compatibility),

which are known to occur in this system (Evans et al. 2012, Oliver & Evans 2014). We estimated egg density from a 5 µl sub-sample of pooled eggs, then added eggs to each aforementioned treatment tube at a density of 15 000 per ml.

After sperm and eggs had been separately exposed to the treatments for 10 min, an aliquot of sperm from each treatment jar was added to the eggs in the treatment tubes equilibrated under the same conditions, at a ratio of 20:1 (sperm density: 300 000 per ml) to give moderate fertilization rates while avoiding ceiling or basement effects (Fitzpatrick et al. 2012), and gently swirled to homogenize the samples. Fertilization was halted after 1 h by adding 1% formalin to each tube. Fertilization rates were assessed under a microscope as the percentage of eggs showing signs of cleavage and/or with polar body formation among approximately 100 haphazardly chosen eggs per replicate (Longo & Anderson 1969). For logistical reasons, fertilization rates were estimated at one time point only (n = 32 males), and sperm motility assays were only undertaken on a subset of the same individual males in the fertilization assays.

Statistical analyses

The CASA assays generated 7 motility parameters, including the curvilinear velocity along the sperm's path (VCL), smoothed average sperm path velocity (VAP), average velocity on a straight line from start to endpoint of the sperm's path (VSL), sperm straightness (STR; the ratio of VSL/VAP), sperm linearity (LIN; the ratio of VSL/VCL), the amplitude of lateral head displacement from the sperm's path (ALH), and the beat frequency of the sperm's flagellum (BCF). As these sperm motility traits are highly correlated, the parameters were condensed with a principal component analysis (PCA) using the 'FactoMineR' package (Lê et al. 2008) in R version 3.2.2 (R Core Team 2016). A separate PCA was performed on the sperm motility traits measured at each time point (Time 1 and Time 2). From each PCA, the sperm parameters were reduced to 2 principal components (PCs) with eigenvalues >1, which together accounted for over 85% of variation in sperm motility at each time point (Table S2 in the Supplement). We used these 2 PCs (PC1 and PC2) in subsequent analyses, and their interpretation is detailed in the 'Results'.

We used linear mixed-effects models to determine the significance of treatment effects on each component of sperm motility (PC1 and PC2 from each PCA) using the 'lme4' package (Bates et al. 2015) in R. The effects of temperature, pH, and their interaction were modeled as fixed effects, while male ID (equivalent to a block effect) and interactions of male ID with treatments were included as random factors. The time elapsed from spawning to motility assessment (min) and year (i.e. corresponding to mussels collected across multiple spawning seasons) were both included as random effects in initial models, but as neither was found to be significant, both terms were removed from final models.

Fertilization rates were analyzed as binomial response variables (proportion of success (fertilized) to failure (unfertilized) for each sample) using a generalized linear mixed-effects model (GLMM) fit by maximum likelihood (Laplace approximation) with logit-link function in 'lme4' (Bates et al. 2015). Temperature and pH were included as fixed effects, while male and interactions between male and treatment effects were included as random factors, along with year.

For all models, Wald chi-square (χ^2) tests were used to assess the significance of fixed effects using the R package 'car' (Fox et al. 2011). To determine the significance of random factors, we performed parametric bootstrapped likelihood ratio tests using the R package 'pbkrtest' (Halekoh & Højsgaard 2014), where each random effect is excluded in turn and the fit of the full model is compared with the model without the random factor (Lynch & Walsh 1998). The bootstrapping method generates multiple estimates of the observed likelihood ratio test statistic (G), calculated as minus twice the difference in log-likelihood and compared with a χ^2 distribution with one degree of freedom (Lynch & Walsh 1998). All non-significant interactions were considered redundant and therefore removed from the final models. For significant treatment interactions, pairwise post-hoc (G)LMMs were run on data subsets to determine which combinations of treatments were significantly different while still taking into consideration significant random effects. All figures were made in R using the package 'ggplot2' (Wickham 2009).

RESULTS

Sperm motility

From the PCAs at both time points, the 3 measures of sperm velocity (VCL, VSL, and VAP) loaded positively on the first principal component (PC1), while BCF loaded negatively on PC1 (Table 1). The second principal component (PC2) was loaded

by STR and LIN (positively) and ALH (negatively) at both time points (Table 1). Thus, high PC1 values represent sperm that swim faster, with a decrease in flagella beat frequency, while high PC2 values indicate straighter-swimming sperm, with a decrease in lateral head displacement. As *Mytilus* spp. sperm swim in a circular motion, measures of BCF and ALH are unlikely to indicate realistic differences in sperm movements or fertilizing ability in this species. Nevertheless, for completeness and consistency, we opted to include all available parameters of sperm motility into the PCAs, but confirm that excluding BCF and ALH from the PCAs did not qualitatively alter the results. Hereafter, sperm motility traits will be referred to as sperm velocity (for PC1 effects) and sperm linearity (for PC2 effects).

There were no significant main effects of pH or temperature on either sperm velocity or sperm linearity at Time 1 (Table 2, Fig. 1). However, we found an interactive effect of pH and temperature on sperm linearity at this initial time point (Table 2). Specifically, we found that sperm swam straighter at the elevated temperature (24°C) than at 18°C, but only under the pH level corresponding to current conditions (pH 8.0; Fig. 1). Furthermore, we found that sperm swam straighter at the most extreme pH level (7.6) than at pH 8.0, but only under the temperature corresponding to current conditions (18°C; Fig. 1). Essentially, sperm swam significantly straighter in the high temperature at pH 8.0 (Wald $\chi^2 = 7.5$, $p < 0.01$), and significantly straighter at pH 7.6 than at pH 8.0 at 18°C (Wald $\chi^2 = 7.7$, $p < 0.01$; Fig. 1). There were no differences in sperm linearity at the lower pH levels at any temperature, and no significant differences at 24°C regardless of pH (Fig. 1).

In contrast to our findings for Time 1, the data for Time 2 revealed significant main effects of temperature on both components of sperm motility and a significant pH effect on sperm velocity (Table 2). Interestingly, we found no evidence for interacting effects of pH and temperature on either component (Table 2). Sperm swam both slower and straighter at the higher temperature compared with the lower one (Fig. 2a,c). The significant pH effect on sperm velocity manifested as slower-swimming sperm at pH 8.0 than at pH 7.8 (Wald $\chi^2 = 7.1$, $p < 0.01$), though not significantly slower than at the lowest pH level (Wald $\chi^2 = 2.3$, $p = 0.10$) and no difference between the lower pH levels (Wald $\chi^2 = 1.3$, $p = 0.30$; Fig. 2b). See Fig. S1 in the Supplement for treatment effects on raw sperm motility parameters relevant to *Mytilus* spp. — VCL, VAP, LIN and STR (Table 1).

The effect of male was significant in all analyses (i.e. at both times for each PC), indicating that there was overall variation

Table 1. Correlations (r) and relative contributions (%) of principal components (PC) from separate principal component analyses on sperm motility parameters at Time 1 and Time 2. Components that contribute over 70% of the weight of each PC are in **bold**

Component	Time 1				Time 2			
	PC1		PC2		PC1		PC2	
	r	%	r	%	r	%	r	%
VAP: smoothed average path velocity	0.9	**20.3**	−0.4	5.9	1.0	**23.2**	−0.3	3.8
VSL: straight-line velocity	0.9	**22.0**	0.3	4.7	0.9	**22.0**	0.3	5.3
VCL: curvilinear path velocity	0.9	**18.5**	−0.5	10.1	0.9	**22.5**	−0.3	4.7
ALH: amplitude of lateral head displacement	0.3	2.6	−0.7	**20.8**	0.4	5.1	−0.5	**12.5**
BCF: beat cross frequency	−0.8	**17.0**	0.3	5.6	−0.8	**14.7**	0.4	6.1
STR: straightness (VSL/VAP)	0.5	7.3	0.8	**30.2**	0.3	2.4	0.9	**39.5**
LIN: linearity (VSL/VCL)	0.7	12.3	0.7	**22.8**	0.6	10.1	0.8	**28.0**

Table 2. Summary of results from linear mixed-effects models on the principal components (PC) of sperm motility at (a) Time 1 and (b) Time 2. Significant p-values are in **bold**. AIC: Akaike's information criterion

Effects	PC1			PC2		
	Wald χ^2/G	p	AIC	Wald χ^2/G	p	AIC
(a) Time 1						
pH	2.60	0.27	647	0.37	0.83	597
Temperature	0.87	0.35	647	1.08	0.30	597
pH × Temperature	–	–	647	7.13	**0.03**	597
−(Male)	11.12	**<0.01**	657	20.30	**<0.01**	617
−(Male × Temperature)	2.50	0.07	648	–	–	–
(b) Time 2						
pH	8.70	**0.01**	640	0.10	0.95	591
Temperature	14.13	**<0.01**	640	11.54	**<0.01**	591
pH × Temperature	–	–	640	–	–	591
−(Male)	8.11	**<0.01**	646	16.74	**<0.01**	608
−(Male × Temperature)	3.66	**0.03**	641	–	–	–

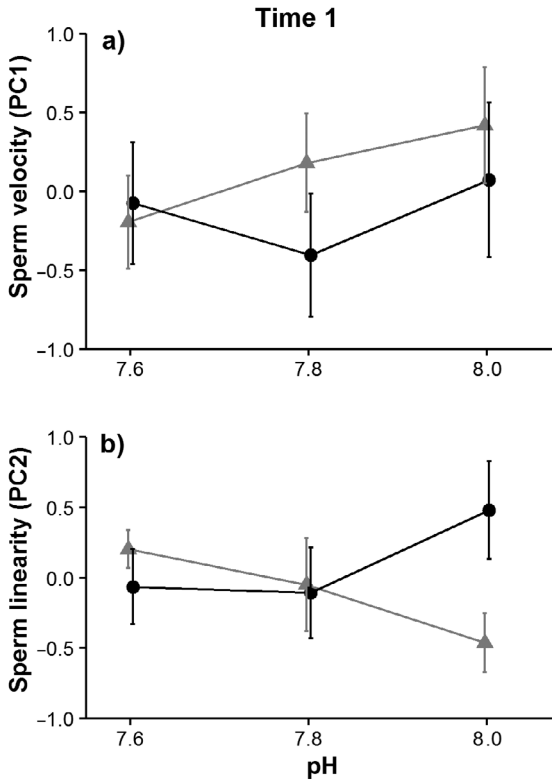

Fig. 1. Interacting effects of temperature and pH on the principal components (PC) of *Mytilus galloprovincialis* sperm motility at Time 1 (20 min in treatment). Light grey lines (▲) indicate 18°C, while black lines (●) indicate 24°C (n = 14; ± SE). No significant main effects or interactions of treatments were detected for sperm velocity (a), although there was a significant interaction between temperature and pH on sperm linearity (b)

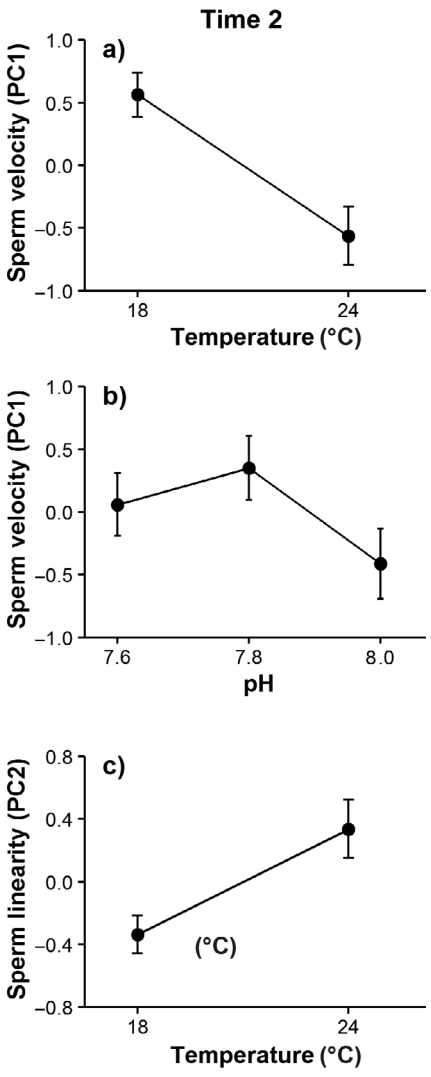

Fig. 2. Effects of temperature and pH on the principal components (PC) of *Mytilus galloprovincialis* sperm motility at Time 2 (2 h in treatment). There are significant main effects of temperature on both sperm velocity (a) and linearity (c) and a significant pH effect on sperm velocity (b), but no evidence for interacting effects of pH and temperature on either component (n = 14; ± SE)

in sperm motility among individuals (Table 2; see Fig. S2 in the Supplement for variation in raw sperm motility parameters across males). There was also a significant male-by-temperature interaction on sperm velocity at Time 2, suggesting that the direction or size of the temperature effect on sperm velocity varied among individual males (Table 2).

By analyzing all data across time points and including 'time' as a fixed effect in an LMM, we confirmed a significant effect of time on both aspects of sperm motility; at Time 2, sperm swam more slowly (Wald $\chi^2 = 52.5$, $p < 0.001$) and straighter (Wald $\chi^2 = 26.1$, $p < 0.001$) than at Time 1.

Fertilization rates

We found significant main effects of both pH and temperature on fertilization rates, and no evidence of any interaction between the treatments (Table 3). Fertilization was significantly higher at the higher temperature (Fig. 3a). The effect of pH was significantly different between the extreme values with fertilization rates higher at pH 8.0 than at pH 7.6 (Fig. 3b).

We also found a significant male effect on fertilization, as well as a highly significant male × temperature interaction, indicating that the direction and/or size of the temperature effect on fertilization rate was not consistent among individual males (Table 3, Fig. S3 in the Supplement). No other variables or interactions were found to influence fertilization rates, except for experimental year, which was kept in the models to control for this variation.

Table 3. Effects of treatment on fertilization rate, analyzed as a binomial response variable using a generalized linear mixed-effects model (GLMM) fit by maximum likelihood (Laplace approximation) with logit-link function. Significant p-values are **bold**. AIC: Akaike's information criterion

Effect	Wald χ^2/G	p	AIC
pH	9.89	**<0.01**	2730
Temperature	15.07	**<0.01**	2730
−(Male)	4.67	**0.03**	2733
−(Male × Temperature)	99.70	**<0.01**	2828
−(Year)	12.66	**<0.01**	2741

DISCUSSION

It has been suggested that ocean warming may buffer the negative effects of ocean acidification in certain cases (Wood et al. 2010, Pecorino et al. 2014; but see Rodolfo-Metalpa et al. 2010, Hale et al. 2011 and Ericson et al. 2012 for examples where elevated temperatures have been shown to exacerbate adverse effects of acidity). Our study on *Mytilus galloprovincialis* offers tentative support for this idea, as we found that the predicted changes to water temperature and pH independently and oppositely affected fertilization rates. That is, higher temperatures led to an increase in fertilization rate, while the lowest pH level resulted in lower fertilization rates. Furthermore, sperm exhibited higher linearity at an elevated temperature than at the current temperature level under current pH conditions, and lowering the pH level did not significantly alter sperm movement patterns in warmer temperatures. This could imply that straighter-swimming sperm have a fitness advantage that leads to higher fertilization rates in this species. However, we also recorded straighter-swimming sperm in the conditions under which fertilization rates were lowest (i.e. 18°C and pH 7.6). We note that while our experimental alteration of pH significantly affected sperm motility and their fertilizing capacity, the mechanism behind the observed effects may be due to changes in other water variables due to the addition of CO_2 (e.g. pCO_2, or carbonate and proton availability).

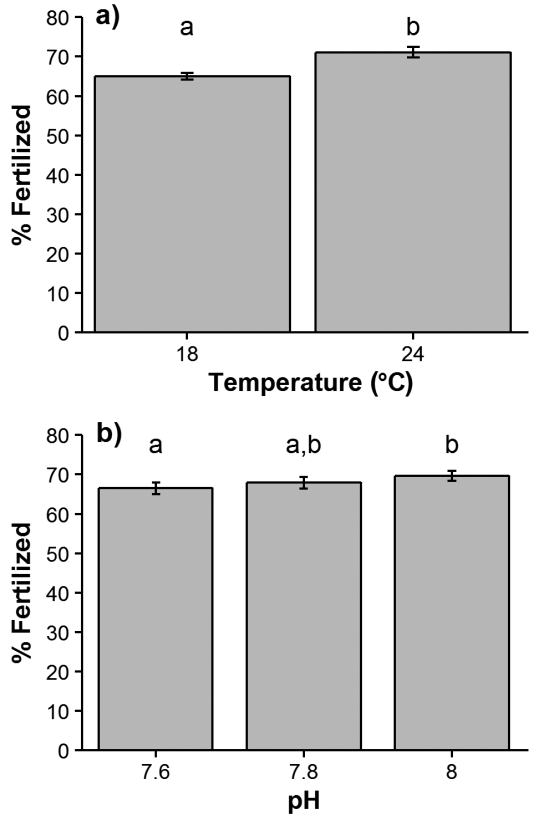

Fig. 3. (a) Temperature and (b) pH effects on *Mytilus galloprovincialis* fertilization rates. There are significant main effects of temperature on fertilization rates, but no evidence for interacting effects of pH and temperature. Letters indicate significant differences (n = 32; ± SE)

It is often assumed across various species that relatively faster-swimming sperm are more successful at fertilizing eggs due to the physics of sperm–egg collision rates (Mita et al. 1984, Levitan 2000, Kupriyanova & Havenhand 2002, Liljedal et al. 2008). However, in *M. galloprovincialis* designating 'successful' sperm motility parameters is more complex as the relationship between sperm motility and fertilization rates is likely to depend on the conditions in which motility is measured. For example, Fitzpatrick et al. (2012) reported that in *M. galloprovincialis*, slower-swimming sperm with a more curved path measured in filtered seawater had the highest fertilization rates. On the other hand, Oliver & Evans (2014) reported that *M. galloprovincialis* sperm swam faster and straighter in the presence of egg chemical cues from more genetically compatible females, thus ultimately leading to elevated fertilization rates by fast-swimming sperm. This apparent contradiction could be explained by chemotaxis, where chemoattractants emitted from unfertilized eggs cause a change in sperm swimming behavior and guide sperm towards eggs (Eisenbach 1999). Fitzpatrick et al. (2012) assessed sperm motility in the absence of egg-derived chemoattractants, and their results therefore may not reflect the motility in the presence of chemoattractants at the time of fertilization. To conserve energy while remaining near spawning females, sperm may restrict their movement by swim-

ming slowly (Stewart et al. 2012) and in tight circles (Liu et al. 2011) until they detect a chemoattractant gradient. After encountering such chemical cues, sperm may then swim faster and in a more linear swimming motion towards the 'chosen' egg (Liu et al. 2011, Oliver & Evans 2014). Therefore, the optimal strategy might be for sperm to swim slowly and in tight circles in the absence of egg chemoattractant cues, but swim faster and straighter in their presence.

We measured sperm motility in the absence of egg chemoattractants and found that sperm swam straighter at a higher temperature and in low pH conditions, independently. In light of the previous discussion on chemotaxis, if straighter-swimming sperm are less likely to remain near compatible eggs after release, this implies a negative effect of higher temperature and low pH on sperm motility. However, any negative impact of high temperature did not result in reduced fertilization rates. The higher fertilization rates at higher temperatures that we observed may be explained by mechanisms other than sperm motility, e.g. decreased seawater viscosity due to the inverse temperature–viscosity relationship (Vogel 1994), an interference with the acrosome reaction (Mita et al. 1984), or an effect of maternal heat shock proteins expressed by the eggs (Diz et al. 2009). Further, the changes in sperm behavior may not have impacted on fertilization rates because we undertook the assays in small, enclosed chambers, leading to a high chance of proximate gamete fertilizations.

We found an overall decrease in sperm swimming speed and linearity after 2 h, regardless of treatment, suggesting that kinematics is energetically costly for *Mytilus* sperm. Furthermore, sperm subjected to a higher temperature over time had significantly decreased swimming velocities and increased swimming linearity compared with those under current temperature conditions. A simple explanation for this pattern is that sperm age faster at warmer temperatures due to increased metabolic rates (Mita et al. 1984); thus, it may be that sperm that can withstand higher temperatures are inherently 'better' sperm (Levitan 2000). However, sperm also swam more slowly at current pH levels than at a lower pH, and we found that under current pH conditions, sperm at the warmer temperature initially swam straighter. Again, this may be an effect of energy conservation, or, in the context of chemotaxis, this could indicate that sperm widen their search pattern if they have not found a compatible egg after this time period. Overall, we confirm that the amount of time sperm spend in potentially stressful environmental conditions can drastically influence sperm motility. A similar study on *M. galloprovincialis* that did not control for the effect of sperm age examined the effect of reduced seawater pH on sperm velocity (not linearity), and found the average sperm swimming speed decreased from 32.2 ± 7.8 µm s^{-1} under control conditions to 23.7 ± 5.6 µm s^{-1} at pH 7.6 (Vihtakari et al. 2013). Interestingly, the sperm velocities reported by Vihtakari et al. (2013) were considerably slow compared with those reported here (131 ± 14.4 µm s^{-1} average velocity across all treatments) and elsewhere (e.g. Fitzpatrick et al. 2012). The reason for this difference is unclear, although the variation in usage times (up to 4.5 h after spawning) in Vihtakari et al. (2013) may explain this difference. As we have shown here, significant differences in sperm motility can be observed over a much shorter period of time (and see, e.g. Purchase et al. 2010), and sperm age can have a major impact on sperm motility (Levitan 2000).

Phenotypic plasticity, or the ability of a genotype to express a range of environmentally dependent phenotypes, is common in organisms inhabiting heterogeneous environments (Ernande & Dieckmann 2004). As *M. galloprovincialis* is likely to encounter a range of environmental conditions during the spawning season, we might expect to see large norms of reaction for many quantitative traits in this species. We suspect that the scope of reaction norms for our measured traits is outside the range of experimental variables we have tested here, as we did not observe a threshold where sperm motility or fertilization ceased. Our results provide evidence for individual variation in sperm motility parameters and fertilization rates among individual males, suggesting that some males exhibit 'intrinsically' better sperm than others. Our analyses also revealed significant temperature-by-male interactions for both sperm traits and fertilization rates. This suggests that the effects of temperature on sperm function may be inconsistent among males, perhaps due to differences in the plasticity of reaction norms, or because 'ideal' environmental conditions for reproduction vary among individuals. While such interacting effects of temperature on sperm 'fitness' may be indicative of heritable genetic variation underlying phenotypic plasticity (i.e. genotype-by-environment interaction), they may also arise through non-genetic factors (e.g. attributable to environmental sources of variance influencing sperm traits, such as male age). We clearly require appropriately designed breeding experiments to determine the extent to which the phenotypically plastic responses uncovered here have a genetic basis (e.g. Rawson & Hilbish 1991, Eads et al. 2012, Lymbery & Evans 2013).

In addition to changes to sperm behavior, ocean warming and acidification may have impacts on female gametes and further impact on chemical communication between gametes. While our study specifically focused on sperm motility, Sung et al. (2014) highlight the importance of considering responses in both sexes. Those authors independently exposed sperm and eggs of the sea urchin *Strongylocentrotus nudus* to a range of reduced seawater pH levels (pCO_2 380–6000 ppmv for 20 min), and found that sperm and eggs exhibited different sensitivities to changes in pH: when eggs were exposed to low pH levels, even as low as pH 7.41, mean fertilization rates remained above 95% (Sung et al. 2014). Conversely, when sperm were exposed, fertilization rates dropped sharply to less than 5% at pH 7.8 and remained similarly depressed at lower pH levels (Sung et al. 2014). As with our findings, their fertilization rates differed with no measured impacts on sperm velocity (which in their study exhibited no decrease under any conditions). As we did not independently expose sperm and eggs to varying environmental conditions, it is unclear whether the results we found for differential fertilization rates under varying environmental conditions were driven by an environmental effect on eggs rather than sperm, or a combination of both. The potential effects that ocean change may have on both the morphology and chemical cues of *Mytilus* spp. eggs would, therefore, be an interesting direction for future research. Furthermore, new techniques are in development that will assist research into the impacts of environmental change on sperm motility and fertilization in a competitive sperm environment (Lymbery et al. 2016).

In conclusion, our results reveal significant environmental effects on sperm motility and fertilization rates in *M. galloprovincialis*. We found complex interactions between pH and temperature on sperm motility (linearity), which in turn may have implications for sperm–egg interactions dependent on changes in sperm swimming behavior (e.g. attributable to sperm chemotaxis; Evans & Sherman 2013). However, we also show that the combined effects of temperature and pH changes on sperm behavior do not necessarily flow through to patterns of fertilization, which reveal less complex effects of temperature and pH on reproductive fitness. While ocean warming may appear to buffer negative impacts of ocean acidification in this study, in combination with prolonged stress of low pH, ocean warming may still have negative consequences for sperm motility. Overall, our findings highlight the need to consider the combined effects of different environmental treatments as well as temporal effects when evaluating gamete responses to environmental stress. We also caution against using only sperm motility as a proxy for reproductive fitness, as our results suggest that they are not directly correlated. *M. galloprovincialis* inhabit a range of coastal intertidal and estuarine waters globally that are already likely to exhibit spatial and temporal fluctuations in temperature and acidity (although not to the same degree of our experimental treatments; Gagliano et al. 2010, Hofmann et al. 2011). Thus, if any negative consequences of climate change can be detected in this tolerant organism, more vulnerable broadcast spawning invertebrates will likely face an uncertain future in changing marine ecosystems.

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LITERATURE CITED

Alavi SMH, Cosson J (2005) Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol Int 29: 101–110

Bates D, Mächler M, Bolker BM, Walker SC (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67: art i01

Brierley AS, Kingsford MJ (2009) Impacts of climate change on marine organisms and ecosystems. Curr Biol 19: R602–R614

Byrne M (2011) Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. Oceanogr Mar Biol Annu Rev 49:1–42

Byrne M (2012) Global change ecotoxicology: identification of early life history bottlenecks in marine invertebrates, variable species responses and variable experimental approaches. Mar Environ Res 76:3–15

Byrne M, Przeslawski R (2013) Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. Integr Comp Biol 53:582–596

Byrne M, Soars NA, Ho MA, Wong E and others (2010) Fertilization in a suite of coastal marine invertebrates from SE Australia is robust to near-future ocean warming and acidification. Mar Biol 157:2061–2069

Caputi N, de Lestang S, Feng M, Pearce A (2009) Seasonal variation in the long-term warming trend in water temperature off the Western Australian coast. Mar Freshw Res 60:129–139

Dias PJ, Fotedar S, Snow M (2014) Characterisation of mussel (*Mytilus* sp.) populations in Western Australia and evaluation of potential genetic impacts of mussel spat

translocation from interstate. Mar Freshw Res 65:486–496
Diz AP, Dudley E, MacDonald BW, Pina B, Kenchington ELR, Zouros E, Skibinski DOF (2009) Genetic variation underlying protein expression in eggs of the marine mussel *Mytilus edulis*. Mol Cell Proteomics 8:132–144
Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO_2 problem. Annu Rev Mar Sci 1:169–192
Eads AR, Mitchell NJ, Evans JP (2012) Patterns of genetic variation in desiccation tolerance in embryos of the terrestrial-breeding frog, *Pseudophryne guentheri*. Evolution 66:2865–2877
Eisenbach M (1999) Sperm chemotaxis. Rev Reprod 4:56–66
Ericson JA, Ho M, Miskelly A, King CK, Virtue P, Tilbrook B, Byrne M (2012) Combined effects of two ocean change stressors, warming and acidification, on fertilization and early development of the Antarctic echinoid *Sterechinus neumayeri*. Polar Biol 35:1027–1034
Ernande B, Dieckmann U (2004) The evolution of phenotypic plasticity in spatially structured environments: implications of intraspecific competition, plasticity costs and environmental characteristics. J Evol Biol 17:613–628
Evans JP, Sherman CDH (2013) Sexual selection and the evolution of egg-sperm interactions in broadcast-spawning invertebrates. Biol Bull 224:166–183
Evans JP, Garcia-Gonzalez F, Almbro M, Robinson O, Fitzpatrick JL (2012) Assessing the potential for egg chemoattractants to mediate sexual selection in a broadcast spawning marine invertebrate. Proc R Soc B 279: 2855–2861
Fitzpatrick JL, Simmons LW, Evans JP (2012) Complex patterns of multivariate selection on the ejaculate of a broadcast spawning marine invertebrate. Evolution 66: 2451–2460
Fox J, Weisberg S, Adler D, Bates D and others (2011) An {R} companion to applied regression, 2nd edn. Sage, Thousand Oaks, CA
Gagliano M, McCormick MI, Moore JA, Depczynski M (2010) The basics of acidification: baseline variability of pH on Australian coral reefs. Mar Biol 157:1849–1856
Galley TH, Batista FM, Braithwaite R, King J, Beaumont AR (2010) Optimisation of larval culture of the mussel *Mytilus edulis* (L.). Aquacult Int 18:315–325
Graham H, Rastrick SPS, Findlay HS, Bentley MG, Widdicombe S, Clare AS, Caldwell GS (2016) Sperm motility and fertilisation success in an acidified and hypoxic environment. ICES J Mar Sci 73:783–790
Gutiérrez JL, Jones CG, Strayer DL, Iribarne OO (2003) Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. Oikos 101:79–90
Hale R, Calosi P, McNeill L, Mieszkowska N, Widdicombe S (2011) Predicted levels of future ocean acidification and temperature rise could alter community structure and biodiversity in marine benthic communities. Oikos 120: 661–674
Halekoh U, Højsgaard S (2014) A Kenward-Roger approximation and parametric bootstrap methods for tests in linear mixed models – the R package pbkrtest. J Stat Softw 59:art i09
Havenhand JN, Schlegel P (2009) Near-future levels of ocean acidification do not affect sperm motility and fertilization kinetics in the oyster *Crassostrea gigas*. Biogeosciences Discuss 6:4573–4586
Hobday AJ, Lough JM (2011) Projected climate change in Australian marine and freshwater environments. Mar Freshw Res 62:1000–1014
Hofmann GE, Smith JE, Johnson KS, Send U and others (2011) High-frequency dynamics of ocean pH: a multi-ecosystem comparison. PLOS ONE 6:e28983
Huang X, Zhuang P, Zhang L, Qiao Z and others (2011) Effects of extracellular environment factors on the motility in Japanese eel spermatozoa. Cell Biol Int 35:505–508
IPCC (2013) Climate change 2013: the physical science basis (eds. Stocker TF, Qin D, Plattner GK, Tignor M and others). Cambridge University Press, Cambridge
Kupriyanova E, Havenhand JN (2002) Variation in sperm swimming behaviour and its effect on fertilization success in the serpulid polychaete *Galeolaria caespitosa*. Invertebr Reprod Dev 41:21–26
Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373:275–284
Lê S, Josse J, Husson F (2008) FactoMineR: n R package for multivariate analysis. J Stat Softw 25:1–18
Levitan DR (2000) Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. Proc R Soc B 267:531–534
Lewis C, Clemow K, Holt WV (2013) Metal contamination increases the sensitivity of larvae but not gametes to ocean acidification in the polychaete *Pomatoceros lamarckii* (Quatrefages). Mar Biol 160:2089–2101
Liljedal S, Rudolfsen G, Folstad I (2008) Factors predicting male fertilization success in an external fertilizer. Behav Ecol Sociobiol 62:1805–1811
Liu G, Innes D, Thompson RJ (2011) Quantitative analysis of sperm plane circular movement in the blue mussels *Mytilus edulis*, *M. trossulus* and their hybrids. J Exp Zool Part A Ecol Genet Physiol 315A:280–290
Longo FJ, Anderson E (1969) Cytological aspects of fertilization in the Lamellibranch, *Mytilus edulis*: I. Polar body formation and development of the female pronucleous. J Exp Zool 172:69–95
Lough JM, Gupta AS, Hobday AJ (2012) Temperature. In: Poloczanska ES, Richardson AJ, Hobday AJ (eds) A marine climate change impacts and adaptation report card for Australia 2012. National Climate Change Adaptation Research Facility, Townsville, p 1–26
Lymbery RA, Evans JP (2013) Genetic variation underlies temperature tolerance of embryos in the sea urchin *Heliocidaris erythrogramma armigera*. J Evol Biol 26: 2271–2282
Lymbery RA, Kennington WJ, Evans JP (2016) Fluorescent sperm offer a method for tracking the real-time success of ejaculates when they compete to fertilise eggs. Sci Rep 6:22689
Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland, MA
Mehlis M, Bakker TCM (2014) The influence of ambient water temperature on sperm performance and fertilization success in three-spined sticklebacks (*Gasterosteus aculeatus*). Evol Ecol 28:655–667
Mita M, Hino A, Yasumasu I (1984) Effect of temperature on interaction between eggs and spermatozoa of sea urchin. Biol Bull 166:68–77
Nakamura M, Morita M (2012) Sperm motility of the scleractinian coral *Acropora digitifera* under preindustrial, current, and predicted ocean acidification regimes. Aquat Biol 15:299–302
Oliver M, Evans JP (2014) Chemically moderated gamete preferences predict offspring fitness in a broadcast

spawning invertebrate. Proc R Soc B 281:20140148
Pearce AF, Feng M (2007) Observations of warming on the Western Australian continental shelf. Mar Freshw Res 58:914–920
Pearce AF, Feng M (2013) The rise and fall of the 'marine heat wave' off Western Australia during the summer of 2010/2011. J Mar Syst 111-112:139–156
Pecorino D, Barker MF, Dworjanyn SA, Byrne M, Lamare MD (2014) Impacts of near future sea surface pH and temperature conditions on fertilisation and embryonic development in *Centrostephanus rodgersii* from northern New Zealand and northern New South Wales, Australia. Mar Biol 161:101–110
Pierrot D, Lewis E, Wallace DWR (2006) MS Excel program developed for CO_2 system calculations. ORNL/CDIAC-105a National Climate Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, TN
Purchase CF, Butts IAE, Alonso-Fernández A, Trippel EA (2010) Thermal reaction norms in sperm performance of Atlantic cod (*Gadus morhua*). Can J Fish Aquat Sci 67: 498–510
R Core Team (2016) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
Raven J, Caldeira K, Elderfield H, Hoegh-Guldberg O and others (2005) Ocean acidification due to increasing atmospheric carbon dioxide. The Royal Society, London
Rawson PD, Hilbish TJ (1991) Genotype-environment interaction for juvenile growth in the hard clam *Mercenaria mercenaria* (L.). Evolution 45:1924–1935
Reynolds RW, Smith TM, Liu C, Chelton DB, Casey KS, Schlax MG (2007) Daily high-resolution-blended analyses for sea surface temperature. J Clim 20:5473–5496
Rodolfo-Metalpa R, Lombardi C, Cocito S, Hall-Spencer JM, Gambi MC (2010) Effects of ocean acidification and high temperatures on the bryozoan *Myriapora truncata* at natural CO_2 vents. Mar Ecol 31:447–456
Schlegel P, Havenhand JN, Gillings MR, Williamson JE (2012) Individual variability in reproductive success determines winners and losers under ocean acidification: a case study with sea urchins. PLOS ONE 7:e53118
Schlegel P, Havenhand JN, Obadia N, Williamson JE (2014) Sperm swimming in the polychaete *Galeolaria caespitosa* shows substantial inter-individual variability in response to future ocean acidification. Mar Pollut Bull 78: 213–217
Sprung M, Bayne BL (1984) Some practical aspects of fertilizing the eggs of the mussel *Mytilus edulis* L. ICES J Mar Sci 41:125–128
Stewart DT, Jha M, Breton S, Hoeh WR, Blier PU (2012) No effect of sperm interactions or egg homogenate on sperm velocity in the blue mussel, *Mytilus edulis* (Bivalvia: Mytilidae). Can J Zool 90:1291–1296
Suchanek TH (1978) The ecology of *Mytilus edulis* L. in exposed rocky intertidal communities. J Exp Mar Biol Ecol 31:105–120
Sung CG, Kim TW, Park YG, Kang SG and others (2014) Species and gamete-specific fertilization success of two sea urchins under near future levels of pCO_2. J Mar Syst 137:67–73
Vihtakari M, Hendriks I, Holding J, Renaud P, Duarte C, Havenhand J (2013) Effects of ocean acidification and warming on sperm activity and early life stages of the Mediterranean mussel (*Mytilus galloprovincialis*). Water 5:1890–1915
Wickham H (2009) ggplot2: elegant graphics for data analysis. Springer-Verlag, New York, NY
Vogel S (1994) Life in moving fluids: the physical biology of flow, 2nd edn. Princeton University Press, Princeton, NJ
Williams ME, Bentley MG (2002) Fertilization success in marine invertebrates: the influence of gamete age. Biol Bull 202:34–42
Wilson BR, Hodgkin EP (1967) A comparative account of the reproductive cycles of five species of marine mussels (Bivalvia: Mytilidae) in the vicinity of Fremantle, Western Australia. Mar Freshw Res 18:175–204
Wilson-Leedy JG, Ingermann RL (2007) Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67:661–672
Wood H, Spicer JI, Lowe D, Widdicombe S (2010) Interaction of ocean acidification and temperature; the high cost of survival in the brittlestar *Ophiura ophiura*. Mar Biol 157: 2001–2013

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