



Variable Individual- and Population-Level Responses to Ocean Acidification

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Population responses to marine climate change are determined by the strength of the selection pressure imposed by changing climate, the genetic variability within the population (i.e., among individuals), and phenotypic plasticity within individuals. Marine climate change research has focused primarily on population-level responses, yet it is at the level of the individual that natural selection operates. We studied individual-level responses of two bivalve species to ocean acidification (OA) at the earliest stage of the life-cycle. We measured sperm activity (swimming speed and percent motility) in the Boreal/Arctic *Macoma calcarea* and the temperate *Mytilus galloprovincialis* in response to two $p\text{CO}_2$ levels (380 and 1000 ppm) at the ambient temperature at the collection site, i.e., 2 and 16°C, respectively. We also assessed sperm longevity under control conditions. Treatment effects on fertilization success were estimated using fertilization models. At the population level, simulated OA reduced *M. galloprovincialis* sperm swimming speed by 30%, percent motility by 44%, and fertilization success by 43%, whereas only sperm swimming speed was significantly affected in *M. calcarea*. Both species showed substantial variability among individuals in response to increased $p\text{CO}_2$. This variability was greatest in *M. galloprovincialis* ranging from non-significant effect to more than 73% reduction in fertilization success in response to OA, whereas *M. calcarea* responses varied from 8% increase in percent sperm motility to 26% reduction in swimming speed. Further, modeled fertilization success was negatively affected by simulated OA in 10 of 13 studied *M. galloprovincialis* males and in three of 10 *M. calcarea* males. We observed sperm longevities (82 h for *M. calcarea* and 25 h for *M. galloprovincialis* on average) far longer than the expected time-frame for efficient fertilization accounting for dilution of gametes. Assuming sperm activity is a heritable trait, natural selection might be a possible way for the studied populations to adapt to near-future OA.

Keywords: individual variability, sperm motility, bivalves, natural selection, *Mytilus galloprovincialis*, *Macoma calcarea*

INTRODUCTION

The rate of anthropogenic climate change may be too rapid for sensitive marine taxa to adapt through new genetic mutations. Hence the response of populations to environmental perturbations will be largely determined by pre-existing genetic variability, the magnitude of the selection pressure caused by changing conditions, and plasticity within individuals (Gienapp et al., 2008; Hoffmann and Sgró, 2011; Reusch, 2014). Therefore, long-term population persistence will not be determined by the average population response, but by the relative responses of different genotypes (Sunday et al., 2011; Schlegel et al., 2012). Genetic variability in traits affected by environmental factors is time-consuming to quantify. Among-individual variability in a measurable trait, however, can give clues as to the initial responses and longer-term adaptability of a population. Average population-level responses to environmental perturbations, on the other hand, may indicate the general sensitivity of a population as well as the magnitude of selection pressure caused by these perturbations. Consequently, population- and individual-level responses provide complementary insights into population vulnerability to future perturbation scenarios.

Most calcifying marine taxa pass through multiple life-stages, each of which may respond differently to selection pressures (Kurihara, 2008; Kroeker et al., 2013). Marine broadcast-spawners shed their gametes directly into the water column, where fertilization takes place (Thorson, 1950), and consequently selection can operate prior to fertilization. Fertilization in broadcast spawners is subject to strong individual selection pressure (Yund, 2000), and depends on complex interactions between gamete traits (Levitan, 2008; Lotterhos and Levitan, 2010; Evans and Sherman, 2013) and the environment (Crimaldi, 2012; Crimaldi and Zimmer, 2013). These interactions determine which gametes—and hence genotypes—meet and fertilize. Hydrodynamic processes lead to a rapid dilution of gametes causing conditions where gamete concentration may become too low for an efficient fertilization (Vogel et al., 1982; Pennington, 1985; Denny and Shibata, 1989; Yund, 2000). The location of an individual within a population of sessile broadcast spawners, spawning synchrony, and the advection of gametes cause high variability in life-time reproductive success among individuals (Denny and Shibata, 1989; Levitan and Young, 1995; Yund, 2000; Himmelman et al., 2008). Nonetheless, gamete traits also affect fertilization success (Vogel et al., 1982; Palumbi, 1999; Styan and Butler, 2000). In particular, percent motility and swimming speed of sperm affect gamete encounter-rate at small scales, favoring males with more active sperm (Figure 1; Levitan et al., 1991; Levitan, 2000; Styan and Butler, 2000; Crimaldi, 2012). Higher swimming speed comes at the cost of reduced longevity, as germ cells have limited energy reserves and few or no homeostatic control mechanisms (Levitan, 2000; Johnson and Yund, 2004). These processes are influenced by pH and temperature (Kupriyanova and Havenhand, 2005; Havenhand et al., 2008; Caldwell et al., 2011), and are presumed to be under genetic control. Consequently, these environmental factors are expected to influence fertilization success and thereby have

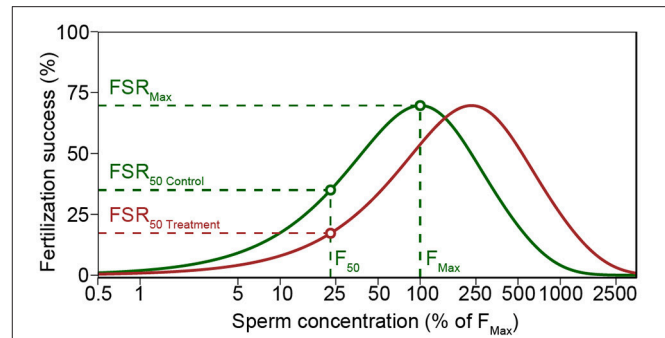


FIGURE 1 | Modeled fertilization success (F_s) for average sperm swimming speed and percent motility of *M. galloprovincialis* in control (green line) and increased $p\text{CO}_2$ treatment (red line). Both control and treatment yield similar maximum F_s (FSR_{Max}), but increased $p\text{CO}_2$ treatment lead to a decreased F_s when sperm concentration is low. Treatment and control F_s was compared using the sperm concentration (F_{50}) that yielded 50% of maximum F_s in control (FSR_{50} Control). Polyspermy block activation time of 10 s was used in the figure to illustrate the decreased fertilization success in high sperm concentrations (see Styan, 1998 and Styan et al., 2008). The figure is based on Schlegel et al. (2012).

substantial influence over the contribution of individuals to the next generation (Havenhand et al., 2008; Parker et al., 2009; Schlegel et al., 2012; Van Colen et al., 2012; Albright and Mason, 2013; Barros et al., 2013).

In this study, we used sperm swimming speed and percent motility as model traits to compare individual- and population-level responses to CO_2 -induced experimental ocean acidification (OA). We used two bivalve species in the comparison: the chalky macoma (*Macoma calcarea*, Gmelin, 1791) is an Arctic to North-Atlantic species (Pedersen, 1994; WoRMS Editorial Board, 2014), whereas the Mediterranean mussel (*Mytilus galloprovincialis*, Lamarck, 1819) has a cosmopolitan distribution (Gosling, 1984; ISSG, 2014). Both of these species are gonochoric free-spawning broadcasters. *Mytilus galloprovincialis* is capable of inhabiting the inter-tidal zone where its gametes might experience a variable pH regime with values that may often reach as low as the predicted open ocean mean values for 2100 (pH drop of ~ 0.3 units, Hofmann et al., 2011; Ciais et al., 2013; Duarte et al., 2013; but see Vihtakari et al., 2013). *M. calcarea*, on the other hand, is solely a sub-tidal species, and the pH experienced by its gametes is presumed to be less variable in the short-term. However, *M. calcarea* is found in the Arctic waters, where OA will be most pronounced during the coming centuries (AMAP, 2013). Sperm activity of *M. galloprovincialis* has been reported to respond negatively to OA levels projected for 2100 (Vihtakari et al., 2013), but to our knowledge, there are no published results on sperm activity or fertilization success for *M. calcarea*. Van Colen et al. (2012) found a significant reduction in average fertilization success with a 0.6 pH unit drop in pH for the related species *Macoma balthica*, but a non-significant effect at pH levels comparable with this study. Both of those studies, however, focused only on population-level responses and did not consider individual variability. The objectives of this study were to (1) identify whether increased $p\text{CO}_2$ affects sperm activity at

the population-level; (2) quantify variation in these effects among individuals and species, and; (3) model the effects on fertilization success using an established fertilization kinetics model (Model S; Styan and Butler, 2000), a revised version of the popular model by Vogel et al. (1982).

MATERIALS AND METHODS

Collection Sites and Animal Handling

Two species of bivalves were used for the experiments: Mediterranean mussel *M. galloprovincialis*, and Boreal/Arctic *M. calcarea*, identified as such according to CO1. Studied species were not protected (Artsdatabanken, 2014). Mussels were obtained from a commercial mussel farm (Manuel Cabrera e Hijo S.C.P), Mahón, Menorca, Spain (11 and 14 of April 2011). The mussel farm uses spat collectors to gather juveniles from natural populations. Water temperature (SST—Sea Surface Temperature) in the region ranges between on average 13.8°C in winter (February) and 25.8°C in summer (August; satellite data from NOAA on www.seatemperature.org) At the time of collection, water temperature was at 15.9°C with 37.5 salinity (psu). The range of pH in seagrass meadows around the Balearic islands is between 0.11 and 0.24 within the canopy as the plants are metabolically very active (Hendriks et al., 2014), while the harvested mussels were in an environment with much more limited fluctuations. Mussels were transported dry but kept humid by air from Menorca to Mallorca. *Macoma* was collected at a depth of 10–25 m, on 29 March and 5 April 2011, at 69°29' 50"N 18°53' 35"E, in Balsfjorden, Northern Norway. Sampling for *Macoma* was conducted outside of protected areas using a Norwegian research vessel (R/V Hyas), and therefore no specific permissions were needed. Water temperature in the fjord ranges between 2.5 and 11.5°C with a salinity range of 14–30 psu (reference sensor in Præbel et al., 2009). *M. calcarea* were packed in coolers, inserted in incisions in wet (seawater) foam to keep them humid and separated them from the cooler packs on top. They were and transported to Mallorca, Spain, by an airplane the day after collection.

Immediately after transport, upon arrival, the bivalves were transferred to temperature-controlled climate rooms set to 16°C (corresponding to the temperature at the collection site) for *M. galloprovincialis* and to 4°C for *M. calcarea*. Water temperature was further lowered to 2°C, the temperature during collection, for tanks holding *M. calcarea* using chillers. Artificial seawater adjusted to the salinity of the collection site (35 ppt for *Mytilus*, 28 ppt for *Macoma*) was made by blending a commercial salt mixture (Instant Ocean Sea Salt) with distilled water. This seawater was thoroughly mixed to ensure a complete dissolution of salt and circulated through a UV lamp for at least 24 h before use to reduce bacterial growth. Adults were kept in tanks with artificial seawater bubbled with ambient air. Measurement of sperm activity was conducted within a week of collection using males that appeared to be in good condition judged by active filtering activity and reaction to tactile stimulus.

Experimental Setup

Spawning

Spawning of *M. galloprovincialis* was induced by a combination of temperature shock (at 22°C) and fluoxetine (Honkoop et al., 1999). In general the mussels started to spawn 30 min after adding fluoxetine, which was then washed away by changing the water to freshly aerated seawater. The mussels were left to spawn for about 30 min. Sperm suspensions from individual males were pipetted from the highest concentrations close to the bottom of the beakers, and transferred to separate Eppendorf tubes. This concentrated solution was mixed with experimental water just before sperm activity experiments (see Vihtakari et al., 2013). Sperm activity measurements for this species were made within 4.5 h of spawning (Table S2). Sperm of *M. calcarea* was collected by strip-spawning (Strathmann, 1987), 10–35 µL of sperm was mixed with 1.5 mL of corresponding experimental water leading to an average suspension of $1.58 \times 10^7 \pm 4.1 \times 10^5$ (SE, $n = 23$) sperm mL⁻¹ across all studied males in the experiment. Sperm activity of *M. calcarea* was measured within 1 h of activation (Table S2). Sperm suspensions were held in Eppendorf tubes in incubators set to the relevant treatment temperature (Table 1).

TABLE 1 | Number of males exposed to the treatments (n) and corresponding seawater parameters.

Species and treatment	n	Measured				Calculated*			
		T	S	pH _{NBS} *	A _T **	pH _T *	pCO ₂	Ω _{Ca}	Ω _{Ar}
M. CALCAREA (MC)									
Control	10	2	28	7.94 ± 0.01	2575 ± 19	8.28	251	3.9	2.4
Acidified	10	2	28	7.53 ± 0.01	2743 ± 14	7.82	852	1.6	1.0
M. GALLOPROVINCIALIS (MG)									
Control	13	16	35	8.09 ± 0.01	3113 ± 22	8.24	321	7.6	4.9
Acidified	13	16	35	7.64	3149 ± 4	7.80	1055	3.3	2.1

Measured seawater parameters: approximate temperature (T, in °C); salinity (S); average pH measured directly from treatment water using sensors (pH_{NBS}, relative to NBS scale, calculated using daily values); and total alkalinity measured from larval cylinders (A_T, in µmol kg⁻¹). Reported error range (±) represents standard error. Given pH value was based on one replicate, if standard error is missing. See Table S1 for number of replicate samples, and Figure S2 for an overview of individual values. Calculated seawater parameters using average pH_T and A_T values after correcting for treatment temperatures (T) using CO2Calc: pH relative to total scale (pH); pCO₂ of seawater (pCO₂, in µatm); calcite saturation state (Ω_{Ca}); and aragonite saturation state (Ω_{Ar}). Species abbreviations are given in brackets after each species. *Corrected for experimental temperature; **Measured at 25°C.

Experimental Water

We used $p\text{CO}_2$ with two levels (380 ppm as control treatment and 1000 ppm as acidified treatment) as a predictor variable to assess the effects of simulated ocean acidification on sperm swimming speed and percentage motile sperm (See **Figure S1** for a schematic overview). Sperm activity was measured at the ambient temperature of the collection site of the bivalves, 2°C for *M. calcaea* and 16°C for *M. galloprovincialis*. The pH of the seawater was manipulated by aerating the seawater with air adjusted to the treatment $p\text{CO}_2$ to each tank separately. Ambient air was collected via aquarium pumps and passed through soda-lime columns. Precise volumes of CO_2 -stripped air and CO_2 gas were administered using mass-flow controllers (Aalborg GFC17) and mixed in a container filled with marbles to achieve the desired $p\text{CO}_2$ concentrations of 380 ppm (control) and 1000 ppm. These values correspond to the annual average atmospheric $p\text{CO}_2$ level of 2005 (Tans and Keeling, 2014) and to the high-end open ocean projected levels for 2100 (Ciais et al., 2013), respectively. pH was measured in the tanks using a spectrophotometer (JASCO 7800) at 25°C following the standard operating procedure (SOP) 6b (Dickson et al., 2007). Total alkalinity (AT) was measured in samples taken for a parallel experiment (see Vihtakari et al., 2013). Sampled seawater was poisoned with 0.2 ml of saturate solution of HgCl_2 (Merck, Analar) to avoid biological alteration, and AT was determined by open cell titration as described in SOP3b (Dickson et al., 2007) with a Metrohm Titrand 808, and Tiamo 2.2 titration software. Certified CO_2 seawater reference material (CRM Batch 101) from the Andrew Dickson lab at UC San Diego was used to evaluate the accuracy of the results. Detailed overview of carbonate chemistry parameters is presented in **Figure S2** and **Table S1**. Temperature-adjusted carbonate chemistry parameters were calculated using average values in **Table 1** and the program CO2Calc (Robbins et al., 2010) (CO_2 constant from Mehrbach et al., 1973, refit by Dickson and Millero, 1987, KHSO_4 formulation from Dickson, 1990).

Water at the corresponding treatment level was collected from 10 L tanks and transferred to 2 L acid-washed and rinsed glass bottles every morning prior to spawning. Additionally to the spectrophotometric pH measurements, pH in the bottles was measured using NIST buffer (pH 4, 7, and 10) calibrated electrodes (Metrohm, 6.0262.100). Sperm activity was measured once or twice per day until no motile sperm cells were observed. Sperm longevity was assessed only under ambient CO_2 conditions, because we were not able to regulate pH in the Eppendorf tubes for the long time period the beyond initial measurements.

Measuring Sperm Activity and Fertilization Assays

Sperm swimming speed was determined from replicated digital video clips of sperm suspension (see **Videos S1, S2**) using four-chamber slides (Leja, Netherlands). The measurement order of replicates (i.e., control first vs. treatment first) was randomized, and the difference between measurements was 14 min at maximum. Sperm swimming speed (VSL—velocity straight line) was measured for each path in 1 s long video

clips using CellTrak 1.3 (Motion Analysis Corp., CA, USA). A motility threshold (definition of when a sperm cell was classified as motile) was determined from sperm speed histograms of single video clips from *control* conditions and set to $10 \mu\text{m s}^{-1}$ for *M. galloprovincialis* and to $9 \mu\text{m s}^{-1}$ for *M. calcaea*. Sperm swimming speed was determined for each video clip by averaging the measured sperm path speeds after excluding non-motile sperm. Percent sperm motility was calculated as a percentage of motile sperm in each video clip. Video clips with fewer than 15 and more than 300 sperm paths were excluded from the dataset as these yielded unreliable measurements. The resulting number of replicates varied between 5 and 11 for start measurements (**Table S2**). See Vihtakari et al. (2013) for further details about sperm activity measurements. Sperm longevity was estimated as the last observation of continuously swimming sperm for each male under *control* $p\text{CO}_2$ treatment (see **Videos S4, S5**).

Numerical Methods

Mean species responses were analyzed by linear mixed-effect models (LMMs) for each species separately using $p\text{CO}_2$ treatment levels as a fixed effect, male as random intercept, and time from spawning as random slope using the following R notation (see Bates et al., 2015 for details):

$$\text{lmer}(y \sim p\text{CO}_2 + (t|\text{Male}))$$

Where y is the response variable (sperm swimming speed, motility or fertilization success) for each species separately, $p\text{CO}_2$ a factor specifying the two $p\text{CO}_2$ treatment levels, t time from spawning and Male a factor specifying each male. Visual examination of non-transformed response variable (percent sperm motility, swimming speed, and modeled fertilization success) histograms revealed that the distributions on treatment level within each male were approximately normal. Analyses were run with *lme4* and *lmerTest* packages (Bates et al., 2015; Kuznetsova et al., 2015) in the R environment (R Core Team, 2015). The statistical significance ($\alpha = 0.05$) of model parameters was estimated primarily from 95% confidence intervals (CIs) for fixed effect slopes, but also expressed using P -values using Satterthwaite approximation for denominator degrees of freedom (SAS Technical Report R-101, 1978).

Effects of increased $p\text{CO}_2$ and variability among and within males was examined by log-transformed response ratios (LnRR), which were calculated for each male separately using video clips as replicates (Hedges et al., 1999; Nakagawa and Cuthill, 2007). Response ratios (RR) were back-calculated as described the following equation and expressed as percentages (modified from Hedges et al., 1999):

$$RR = 100 \exp \left[\ln(\bar{X}_{\text{Treatment}}) - \ln(\bar{X}_{\text{Control}}) \right] \%$$

Where \bar{X} is the average response. The 95% confidence intervals for response ratios were calculated using t-distribution to account for low sample size (modified from Hedges et al., 1999):

$$100 \exp \left(\text{LnRR} - t_{0.05} \sqrt{v} \right) \% \leq RR \\ \leq 100 \exp \left(\text{LnRR} + t_{0.05} \sqrt{v} \right) \%$$

where $t_{0.05/2}$ is the 95% critical cut point for a t -distribution with degrees of freedom $n_{\text{control}} + n_{\text{treatment}} - 1$, where n is number of replicates, and v combined variance for treatment and control calculated using following equation from Hedges et al. (1999):

$$\frac{SD_{\text{Treatment}}^2}{n_{\text{Treatment}}\bar{X}_{\text{Treatment}}^2} + \frac{SD_{\text{Control}}^2}{n_{\text{Control}}\bar{X}_{\text{Control}}^2}$$

Where SD is standard deviation. The equations above are demonstrated as R code in Supplementary Material (Datasheet S1). Mean species response ratios were extracted from the linear mixed effect model results using confidence intervals for the fixed effect slope to calculate response ratio confidence intervals.

Fertilization success (Fs) was calculated using Model S (Styan, 1998; Styan and Butler, 2000; Styan et al., 2008), a revised version of the model by Vogel et al. (1982) (Figure 1). For all comparisons, model parameters were set to be constant with the exception of sperm swimming speed and egg diameter [61 μm for *M. galloprovincialis* (Sedano et al., 1995), and 95 μm for *M. calcarea* (Oertzen, 1972)]. The model was run using sperm swimming speed from each replicate video clip after multiplying the initial sperm concentration (S_0) by the proportion of motile sperm in each replicate. Afterwards, a mean fertilization model was calculated for each male and treatment. The sperm concentration that yielded 50% of maximum F in control treatments (F_{50} in Figure 1) was chosen to compare fertilization success among treatments. Response ratios were calculated as described above. The fertilization modeling is demonstrated using one of the males as an example in the R code included in Supplementary Material (Datasheets S1, S2).

RESULTS

Water Chemistry

Difference in pH between low (380 ppm, control hereafter) and high $p\text{CO}_2$ (1000 ppm, acidified hereafter) treatments was stable throughout the study, the average difference in pH_T being 0.42 for *M. calcarea* at 2°C and 0.43 for *M. galloprovincialis* at 16°C (Figure S2, Table 1). Average total alkalinity (A_T) was 2659 $\mu\text{mol kg}^{-1}$ or *M. calcarea*, and ranged from 2539 to 2770 $\mu\text{mol kg}^{-1}$ (Figure S2, Table 1). Average A_T for *M. galloprovincialis* was 3130 $\mu\text{mol kg}^{-1}$ ranging between 3010 and 3164 $\mu\text{mol kg}^{-1}$. Total alkalinity was significantly higher in acidified treatments compared to control treatments for *M. calcarea* (168 $\mu\text{mol kg}^{-1}$ on average; Welch Two Sample t -test: $n = 6$, $t = -7.098$, $\text{df} = 3.761$, $p = 0.002$; Figure S2). There was no significant difference in A_T values between acidified and control for *M. galloprovincialis* (Welch Two Sample t -test: $n = 12$, $t = -1.640$, $\text{df} = 5.289$, $p = 0.159$; Figure S2).

Fertilizing Capacity of Sperm

Fertilization experiments were conducted to examine whether the studied sperm was capable of fertilizing eggs. Sperm from every *M. galloprovincialis* fertilized eggs successfully and the eggs developed to early trochophore stage, at which point the experiment was terminated. In contrast, *M. calcarea* did not

produce viable embryos. The eggs of this species, which were also stripped, were clearly irregular and immature. Nonetheless, both of the two tested *M. calcarea* males successfully fertilized eggs, although the eggs did not develop beyond the 8-cell stage.

Acidification Responses

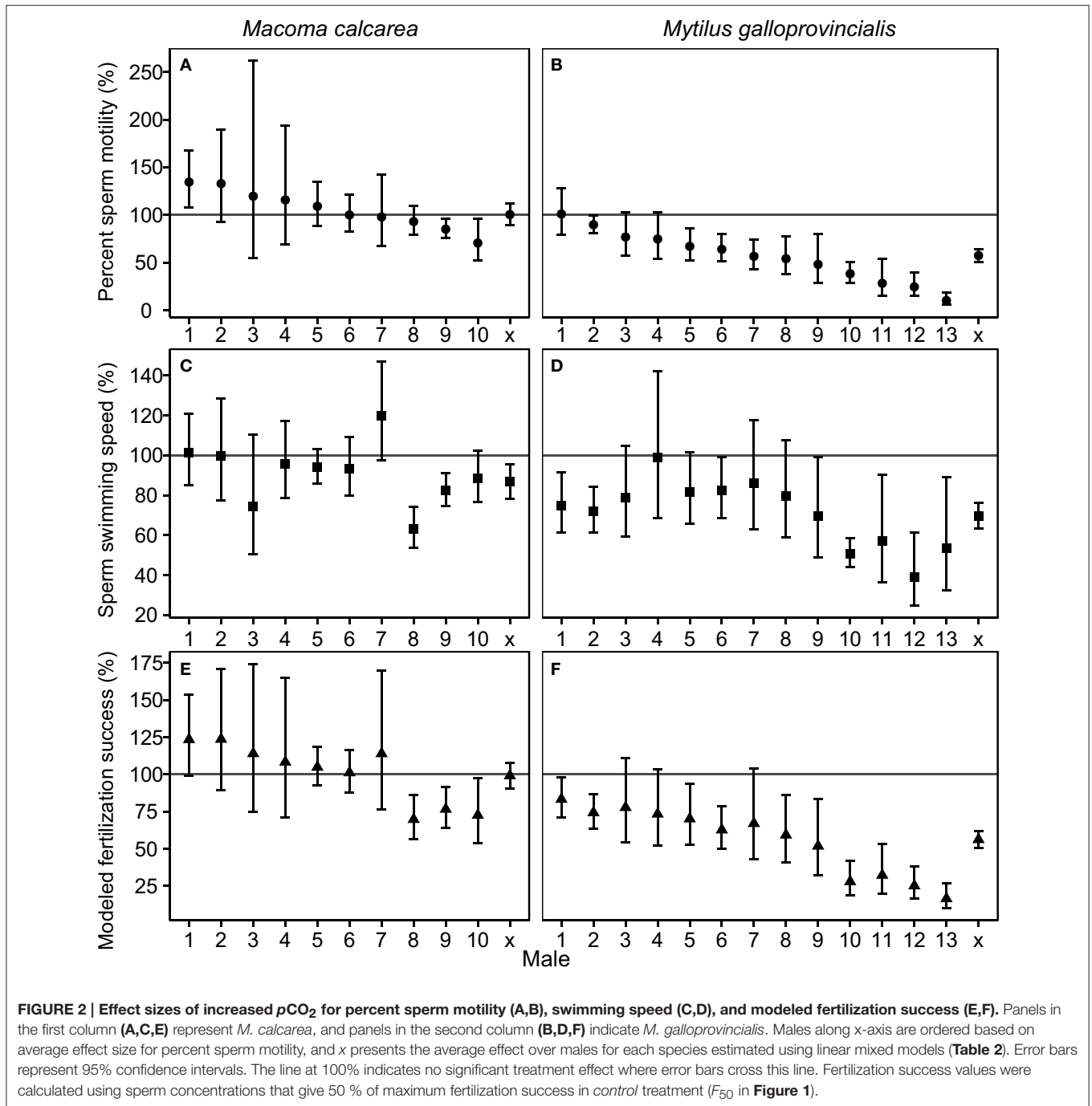
The population-level effects of increased $p\text{CO}_2$ on percent sperm motility, and modeled fertilization success of *M. calcarea* were not significant, while these effects for *M. galloprovincialis* were significantly negative (Figure 2, Figure S3). There was substantial variability between males in response to increased $p\text{CO}_2$ in both species (Figure 2). This variability was largest in *M. galloprovincialis*, with response ratios varying from a non-significant 100.5% effect to a tenfold reduction ($R = 10.1\%$, CIs 5.4–18.7%) in percent sperm motility, and from a non-significant 98.6% to a two-fold reduction ($R = 39.0\%$, CIs 24.5–61.4%) in sperm swimming speed (Figure 2, Videos S1, S2). Significantly negative $p\text{CO}_2$ effects on modeled fertilization success were observed in three of 10 *M. calcarea* males (Figure 2E). One *M. calcarea* male showed a positive effect that was marginally non-significant ($R = 123.5\%$ CIs 99.3–153.5%). Increased $p\text{CO}_2$ had a significant negative effect on modeled fertilization success in 10 of 13 *M. galloprovincialis* males (Figure 2F). The response ratios varied from 15.3% (CIs 10.3–22.6%) to 83.8% (CIs 67.0–104.7%).

Sperm Longevity

Sperm longevity was estimated only in control $p\text{CO}_2$ conditions and at the respective ambient temperature for each species (Table 1, Figure 3, Videos S3, S5). Average sperm longevity for *M. calcarea* [81.7 ± 11.3 h (SE, $n = 9$)] was longer than that for *M. galloprovincialis* [24.7 ± 3.5 h (SE, $n = 13$)]. Males demonstrated variability in sperm longevity: four *M. galloprovincialis* males had a markedly shorter sperm longevity (~ 10 h) than the rest (25.2–46.8 h, Figure 3, Table S3). In *M. calcarea*, sperm longevity among males ranged between 22.5 and 142.6 h (Table S3).

DISCUSSION

We observed substantial variability among individuals in response to simulated ocean acidification in both studied species (Figure 2). Fertilization modeling predicted a significant negative effect of acidification on fertilization success for the majority of *M. galloprovincialis* (10 of 13, Figure 2F), but only for 3 of 10 *M. calcarea* males (Figure 2E). Among-individual variability in response to OA comparable to that found in *M. calcarea* has been reported for a sea urchin (Schlegel et al., 2012), and an oyster (Havenhand and Schlegel, 2009), whereas similar strong variability as seen in *M. galloprovincialis* was previously observed in a polychaete (Schlegel et al., 2014). These results suggest that among-individual variability is likely the norm, rather than an exception, and represents adaptive potential enabling organisms to cope with environmental change. Sperm from males less affected by acidification are likely better adapted to fertilize eggs in a future ocean, and therefore—assuming these beneficial sperm traits are heritable—the offspring of these males will become more abundant in the gene pool (Schlegel et al., 2014).



Simulated ocean acidification (increased $p\text{CO}_2$ at 16°C) had substantial and negative population-level effects on *M. galloprovincialis* sperm activity, as previously described in Vihtakari et al. (2013), and on modeled fertilization success (Figure 2F, Table 2). In contrast, significant population-level reductions were observed only in sperm swimming speed in *M. calcaria* at 2°C (Figures 2A,C,E). Interestingly, the response was more negative in the intertidal *M. galloprovincialis*, whose gametes may already occasionally experience pH values as low

as projected for 2100 (Ciais et al., 2013; Duarte et al., 2013; Hendriks et al., 2014), than in *M. calcaria* (Figure 2). This could indicate that variable conditions might maintain a higher level of phenotypic variation in the population as several environmental stressors might act simultaneously (Sunday et al., 2014), although data on more species are needed to draw such conclusions. In many marine invertebrates sperm are stored immotile in the acidic environment of the testis, which inhibits sperm respiration and metabolism (Caldwell et al., 2011; Schlegel et al., 2012;

Alavi et al., 2014). The increase in pH upon release is partly responsible for activation of mitochondria in sea-urchin sperm (Christen et al., 1982). Although, sperm activation in marine bivalves appears to be more complex, pH is likely to contribute to sperm activation (Alavi et al., 2014). A weaker pH shock due to lower environmental pH could therefore partly explain the lower percentage of motile sperm in the increased $p\text{CO}_2$ treatment.

Van Colen et al. (2012) also found a non-significant decrease in fertilization success of the related species *M. balthica* at a similar pH reduction ($\Delta\text{pH} \sim 0.3$ units), and a significant 11% reduction in fertilization success at ΔpH of 0.6 units. It is possible that the population-level fertilization success in *M. calcaea* could also be negatively affected by such pH reductions since our modeling results indicate that some individuals were negatively

affected by the studied 0.3 unit drop in pH (Figure 2E), and sperm swimming speed was negatively affected on population level (Figure 2C). Other acidification effects on fertilization success of bivalves include a significant reduction for *Saccostrea glomerata* starting from a $p\text{CO}_2$ level projected for the end of this century (600 ppm; Parker et al., 2009), and a reduction for *Crassostrea gigas* at a far-future ΔpH of 0.7 units (Barros et al., 2013). Havenhand and Schlegel (2009), on the other hand, found no significant population-level effects on sperm activity or fertilization success of *C. gigas* for a pH reduction similar to that used in this study (~ 0.35 units)—a result that is consistent with Barros et al. (2013). Negative population-level effects of OA on fertilization success appear to be common for echinoderms (Havenhand et al., 2008; Morita et al., 2010; Schlegel et al., 2012; Uthicke et al., 2013), corals (Morita et al., 2010; Albright and Mason, 2013), and a polychaete (Schlegel et al., 2014) among free-spawning marine invertebrates, however a positive effect of OA on sperm activity of a sea urchin has also been observed (Caldwell et al., 2011). A recent review concludes that fertilization in benthic marine invertebrates could be relatively robust to OA (Byrne and Przeslawski, 2013). Considering the negative effects that have been reported for a wide range of benthic taxa, and the results of this study, this might not be a good generalization. Although, it is clear that population-level effects of OA on fertilization kinetics of marine invertebrates vary among species, populations, and gamete concentrations, our results together with the literature suggest that OA is likely to decrease the sperm performance in a range of benthic marine invertebrates, potentially resulting to a lower fertilization success in sperm limited low density populations (Levitan and Petersen, 1995; Styan, 1998; Levitan, 2000; Albright and Mason, 2013). The consequences of these reductions are unpredictable considering the complex fertilization kinetics in marine broadcast spawners (Denny and Shibata, 1989; Levitan and Young, 1995; Yund, 2000; Crimaldi and Zimmer, 2013; Evans and Sherman, 2013).

Experimental studies are subject to biases that complicate projecting future population responses (Vihtakari, 2014). Here the lack of adult acclimation and the use of artificial seawater might have influenced results. Adult acclimation in experimental conditions might affect the responses of offspring to ocean

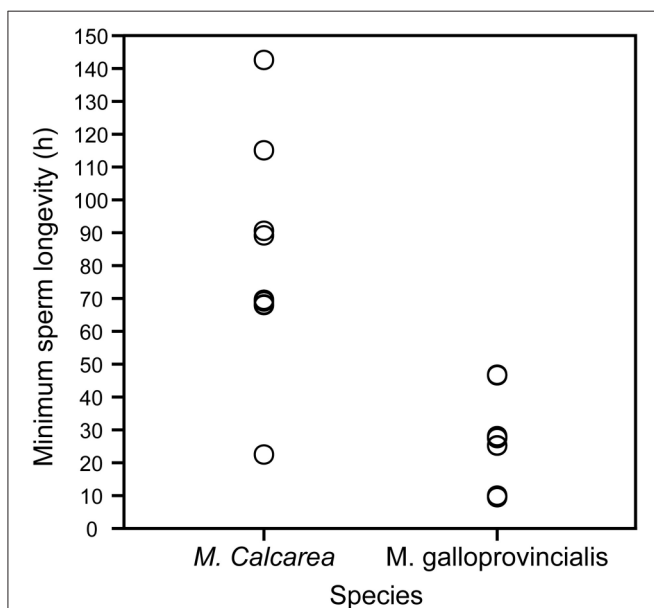


FIGURE 3 | Sperm longevity of *M. calcaea* and *M. galloprovincialis*. Each open circle represents a time (h) for the last observation of continuously swimming sperm for an individual male.

TABLE 2 | Linear mixed-effects model results.

Species	Variable	Intercept (control)		Slope (treatment)		Random effect variance				Satterthwaite approximations					
		β_0	95% CIs	β_1	95% CIs	Intercept	Time	Int-time	Residuals	SE	df	t	p		
MC	Motility	34.8	24.8	44.7	0.1	-3.8	4.0	634.4	2335.3	-968.4	88.8	2.0	27.0	0.05	0.959
	Speed	39.3	32.4	46.2	-5.2	-8.6	-1.8	808.3	2718.7	-1379.1	53.2	1.7	44.6	-2.99	0.004
	Fertilization	50.3	46.6	54.0	-0.4	-4.7	3.8	25.5	8.6	-14.8	190.0	2.2	143.7	-0.20	0.844
MG	Motility	46.7	41.0	52.5	-19.9	-23.0	-16.9	2347.0	151.6	-596.6	151.2	1.6	237.5	-12.77	< 0.001
	Speed	31.0	28.2	33.8	-9.4	-11.4	-7.4	292.1	13.9	-63.8	64.7	1.0	238.2	-9.25	< 0.001
	Fertilization	49.4	46.3	52.4	-21.6	-24.4	-18.8	74.2	7.5	-20.9	118.0	1.4	220.7	-15.16	< 0.001

Treatment $p\text{CO}_2$ was used as fixed effect, male and time from spawning as random effects. The model was run separately for each species (MC, *M. calcaea*; MG, *M. galloprovincialis*). β_0 indicates the intercept for control (in %, $\mu\text{m s}^{-1}$ and % for sperm motility, swimming speed, and modeled fertilization success, respectively) and β_1 the mean effect (slope) compared to β_0 . P-values were estimated using Satterthwaite approximations.

acidification through transgenerational plasticity (Miller et al., 2012; Parker et al., 2012; Dupont et al., 2013) as gametes inherit nutrition and other bioactive materials from their parents (Munday et al., 2013; Salinas et al., 2013). Since we did not acclimatize studied males, we cannot assess the possible consequences of such effects. Furthermore, the use of artificial seawater led to 500–700 $\mu\text{mol kg}^{-1}$ higher total alkalinity (A_T) than measured for the Mediterranean for *M. galloprovincialis* (Millero et al., 1979; Gazeau et al., 2005; Schneider et al., 2007; but see Vihtakari et al., 2013), and 200–400 $\mu\text{mol kg}^{-1}$ higher than published A_T values for the Barents Sea (Chierici et al., 2012) for *M. calcarea*. Despite this, measured pH and calculated seawater $p\text{CO}_2$ values were within the range projected for 2100 in the *acidified* treatment (Gattuso and Lavigne, 2009; Ciais et al., 2013) and within the range of current values for the *control* treatment (Millero et al., 1979; Murphy et al., 2001). Temperature could also influence the responses to OA (Byrne et al., 2009, 2010; Parker et al., 2009; Vihtakari et al., 2013), and therefore the observed responses could have been different had we considered the combined effects of OA and global warming in our experimental design. Manipulating temperature would be a logical next step, as OA and temperature will change both in the future. Results of experimental studies should be interpreted with the understanding that no single experiment can accurately assess the consequences of future perturbations to biota under climate change. Instead, these experiments add to the growing body of evidence that ocean acidification might have complex consequences to marine ecosystems.

Sperm longevities estimated for *M. calcarea* (82 h at 2°C on average, **Figure S3**) and *M. galloprovincialis* (25 h at 16°C) in this study are remarkably long considering that effective fertilization is expected to occur within minutes from spawning due to rapid dilution of gametes (Pennington, 1985; Denny and Shibata, 1989; Yund, 2000). Long lasting sperm has been previously reported in bivalves: Powell et al. (2001) found that sperm of the Antarctic bivalve *Laternula elliptica* were capable of fertilizing eggs more than 90 h after spawning at $\sim 0^\circ\text{C}$. The highest sperm longevity values in this study (4–6 days for *M. calcarea*) were of a similar order of magnitude than those found by Alavi et al. (2014) for Pacific oyster *C. gigas* (4–6 days), Manila clam *Ruditapes philippinarum* (~ 7 days), and Japanese scallop *Patinopecten yessoensis* (2–4 days), all at room temperature. Previous longevities reported for near-shore bivalves are considerably shorter *Mytilus edulis*: >5 h (Levy and Couturier, 1996); *Cerastoderma edule*: 4–8 h (André and Lindegarth, 1995). Sperm longevity, i.e., the time that sperm remain motile (**Figure 3**, **Videos S1**, **S3–S5**), however, does not measure the fertilization capability of sperm and consequently might lead overestimating the actual effectiveness of sperm to fertilize eggs. In this study, sperm reached the immotile state after an average of 9 days for *M. calcarea* and within 38 h for *M. galloprovincialis*. Filming sperm motility requires relatively high sperm concentrations. The extreme longevities could partly be explained by the high concentration of studied sperm, which could lead to sperm being restricted by space and therefore not swimming as actively as in the field (Johnson and Yund, 2004). Also reduced motility caused by sperm cells sedimenting at the

bottom of Eppendorf tubes could decrease the amount of energy consumed compared to the natural environment. Nevertheless, bivalve sperm might stay active far longer than the time-frame of efficient fertilization (André and Lindegarth, 1995; Powell et al., 2001; Alavi et al., 2014) due to hydrodynamic processes leading to a rapid dilution of gametes (Denny and Shibata, 1989; Levitan and Petersen, 1995; Yund, 2000). This is an interesting observation and poses a question whether sperm longevity could be of an advantage in certain conditions. Powell et al. (2001) suggested that long-lived sperm could increase the effectiveness of synchronized mass spawning events in *L. elliptica* by allowing time for sperm densities to reach the levels needed for high fertilization success. Although this hypothesis could also apply for *M. galloprovincialis* and *M. calcarea*, there is currently not enough field-data to identify the potential reasons for sperm remaining active longer than the dilution of gametes.

CONCLUDING REMARKS

Ocean acidification negatively affected sperm swimming speed, percent motility and modeled fertilization success in *M. galloprovincialis* on a population-level. There was a substantial among-male variability in these responses varying from statistically non-significant to a 90% reduction among *M. galloprovincialis* males. Among-male variability in *M. calcarea* males ranged from significantly positive in percent sperm motility to a $>26\%$ reduction in swimming speed.

Population-level responses may be useful for detecting traits that are vulnerable to climate change, but they tell us little about the adaptive capacity of a population to future conditions. The key determinant for a species' success in the future ocean is the extent of genetic variability in traits that are susceptible to climate change. Inter-individual variability could, thus, mitigate the effects of climate change on future populations (Sunday et al., 2011; Schlegel et al., 2014). Inter-individual variability is likely the norm, rather than an exception, and the responses of populations in the future may therefore differ from the average response of a population today. Nonetheless, the long-term effects of strong selection for acidification on population (and species) viability are difficult to predict. Importantly, there are no data linking robustness of sperm performance under environmental change in a single parameter, such as pH, to robustness of the resulting individual during the remainder of its life-cycle. Reduced individual-variability through selection may have negative consequences for adaptability to ocean acidification during other phases of the life-cycle, or other stressors altogether. The possibility that gamete, larval, and adult robustness are co-evolved is exciting and deserves further attention.

AUTHOR CONTRIBUTIONS

IH was in charge of the experimental water treatments, and analysis of the carbonate system, MV performed sperm measurements, and analyzed the data, PR, JH, IH, and MV designed the experiment and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmars.2016.00051>

Table S1 | Water chemistry sample overview over studied males. The first two letters in Male column represent the species (see **Table 1** for the species abbreviations) and the number the male number from **Figure 1**. S, measured salinity in treatment tanks; pH_{NBS} , sensor measurement directly from treatment water; pH_T , spectrophotometer measurement from storage tanks; and A_T , total alkalinity measurement from larval cylinders. A number in pH_{NBS} , pH_T , and A_T columns represent the number of replicate measurements. Missing values indicate that measurement was not conducted. Results of the measurements are presented in **Table 1**.

Table S2 | Experimental data averaged over males. The first two letters in Male column represent the species (see **Table 1** for the species abbreviations) and the number the male number from **Figure 1**. pCO_2 , air pCO_2 level; T, temperature ($^{\circ}\text{C}$); pH_{NBS} , pH in NBS scale; Time, average time from spawning when measurements were conducted (min); C, sperm concentration at the beginning of measurements (sperm μL^{-1}); and n, number of replicate film clips included in the analyses. Average and standard deviation for percentage sperm motility (Motility, %), sperm swimming speed (Speed, $\mu\text{m s}^{-1}$), and number of sperm paths (Paths) per replicate film clip are given in x and σ columns respectively.

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Table S3 | Longevity data for Figure 3. Columns from the left: Species, corresponding species (see **Table 1** for the species abbreviations); Male, the male number from **Figure 1**, and x indicates species average; Time, time of the last observation of continuously swimming sperm in hours.

Video S1 | Video clip of *M. galloprovincialis* (MG10) sperm under control conditions during initial sperm activity measurements. The video clip shows continuously swimming sperm in good condition.

Video S2 | Video clip of *M. galloprovincialis* (MG10) sperm under increased pCO_2 during initial sperm activity measurements. Sperm is clearly affected by the treatment (compare with **Video S1**).

Video S3 | Video clip of *M. galloprovincialis* (MG3) sperm under control conditions 46 h after induced spawning. The video clip shows several continuously swimming sperm cells.

Video S4 | Video clip of *M. calcaea* (MC5) sperm under control conditions during the initial measurements. The video clip shows several continuously swimming sperm cells in good condition.

Video S5 | Video clip of *M. calcaea* (MC5) sperm under control conditions 115 h after extraction of gametes. One cell adjacent to the lower left corner is swimming continuously. Many cells are still swimming, although not as continuously as in **Video S4**, and therefore not recorded as “continuously swimming sperm.”

Datasheet S1 | Code S1.

Datasheet S2 | Code S2.

Figure S1 | Experimental setup. On the left gas-mixture, with CO_2 bottle, sodalime, Mass Flow Controllers (MFCs) producing gas mixtures with 380 and 1000 ppm CO_2 . In the middle storage conditions for *M. calcaea* and *Mytilus edulis* in separate climate rooms and on the right conditions in which the water used during the video trials was stored. Green for 380 ppm and red for 1000 ppm CO_2 gas/water.

Figure S2 | Water pH (A,B) and total alkalinity (AT; C) during the study. Circles represent *control* and triangles *acidified* treatments. (A) pH sensor values measured directly from treatment water (relative to NBS scale). (B) Water pH values measured from storage tanks using spectrophotometer (relative to total scale). (C) Water AT (mmol kg^{-1}) values measured from larval cylinders. Vertical lines represent days with sperm activity assessments. Individual males from **Figure 1** are specified on top of the graph.

Figure S3 | Raw data used to calculate the effect sizes (Figure 2). (A,B) percent sperm motility, (C,D) sperm swimming speed, and (E,F) modeled fertilization success. Panels in the first column (A,C,E) represent *M. calcaea*, and panels in the second column (B,D,F) indicate *M. galloprovincialis*. Lines between points mark individuals and error bars show standard error of mean. Green indicates control pCO_2 treatment, while red increased pCO_2 treatment.

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