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Original Article

Simulated diurnal pH fluctuations radically increase *variance* in—but not the *mean* of—growth in the barnacle *Balanus improvisus*

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Shallow coastal waters are characterized by substantial diurnal fluctuations in pH, especially in nearshore environments. The biological effects of ocean acidification in combination with these natural fluctuations have received relatively little attention to date. We exposed multiple batches (≈ different genotypes) of newly settled barnacles, *Balanus improvisus*, to constant pH under "control" (pH = 8.1) or "stable acidified" (pH = 7.7) conditions, as well as a treatment that simulated the maximum diurnal pH fluctuations seen in the nearshore habitats where this barnacle lives (± 0.2 pH units), superimposed on the stable acidified treatment ("fluctuating acidified"; 7.5 ≤ pH ≤ 7.9). We found that fluctuating acidification had no effect on *mean* response in growth and shell mineralogy, but caused an ~20-fold increase in *variance* of responses, compared with stable acidification. In contrast to these results, we found no effect of fluctuating acidification on variances of response ratios for barnacle survival and shell strength. Similarly, mean survival did not vary significantly with pH. However, we observed a strong negative effect of stable and fluctuating acidification on mean shell strength. Our finding that barnacles respond differently to fluctuating pH than to stable low pH indicate the importance of including fluctuating acidification treatments when studying species that live in variable environments. Importantly, because phenotypic variance is the raw material for natural selection, and thus lays at the heart of evolutionary responses to environmental variability and change, our findings also highlight the need to study changes in variance of—as well as mean—responses to changing ocean climates.

Keywords: crustacea, effect size, natural fluctuations, ocean acidification, penetrometry, response ratio.

Introduction

Shallow marine habitats experience substantial natural fluctuations in seawater chemistry that are typically far greater than those in the open oceans (Hofmann *et al.*, 2011; Duarte *et al.*, 2013). Seasonal changes in river run-off, upwelling, and shifts in primary and secondary production can cause pH fluctuations of up to—and sometimes more than—1 unit in these habitats (Blackford and Gilbert, 2007; Wootton *et al.*, 2008; Hofmann *et al.*, 2011; Pansch *et al.*, 2012). Even on much shorter diurnal time-scales, photosynthesis and respiration can cause very large pH excursions (Agnew and Taylor, 1986), although the effects of diurnal pH fluctuations are more typically 0.2–0.4 pH units in shallow habitats (Wootton *et al.*, 2008; Hofmann *et al.*, 2011; Cornwall *et al.*, 2013; Challener *et al.*, 2015).

The effects of diurnally fluctuating pH on organisms have been addressed in comparatively few studies to date; indeed, the great majority of published ocean acidification (OA) work has investigated the effects of constant levels of OA (which are well established to have generally, though not uniformly, negative effects across a variety of traits; Kroeker *et al.*, 2013). Clearly, the environmental relevance of using constant pH levels to determine the effects of OA on organisms that live in a fluctuating environment is limited, not only because future ocean pH is projected to vary at least as much as—and perhaps even more than—it does today (Takeshita *et al.*, 2015), but also because selection tends to be stronger under extreme, rather than average conditions (Darwin, 1859; Hoffmann and Parsons, 1997).

Recent reviews of the effects of environmental fluctuation on organisms have demonstrated that: (i) environmental variance

can lead to fluctuating selection pressures, which prevent species from tracking phenotypic optima (Kopp and Matuszewski, 2014); and (ii) changes in both the mean and variance of ecologically important environmental variables—such as might be expected under climate change—can have very different effects on organism growth rates (Lawson *et al.*, 2015). Experimental investigations of the effects of fluctuating environmental variables have tended to focus on factors other than OA (e.g. Stenseth *et al.*, 2002), and the few studies that have investigated organism responses to fluctuating acidification have found varying responses. Alenius and Munguia (2012) studied the effects of "low stable" (ΔpH = −0.4 units), "low variable" (ΔpH = −0.4 ± 0.1 units) and "control" (ambient pH) treatments on the intertidal isopod *Paradella dianae*, and found the strongest negative effects in "low variable" treatments for the means of most (but not all) response variables (Alenius and Munguia, 2012). Stronger negative effects of fluctuating rather than stable acidification were also observed for mean growth and recruitment rates of coralline algae (Cornwall *et al.*, 2013). Other authors have found effects of fluctuating acidification (on calcification of coralline algae, Johnson *et al.*, 2014; or growth of salmon, Ou *et al.*, 2015), or even positive effects on calcification of corals (Dufault *et al.*, 2012; Comeau *et al.*, 2014) and recruitment of corals (Dufault *et al.*, 2012). Always, these publications have highlighted the need for more investigations of the effects of environmentally relevant pH fluctuations superimposed on projections of future OA. There is now a pressing need to determine whether the effects of diurnal naturally fluctuating acidification are similar to, or different from, the relatively well-documented responses of marine organisms to different levels of stable OA.

Importantly, although the aforementioned studies reported the effects of variable environment on mean organism response and its variance, the discussions focused on differences in *mean* response. This is a universal practice, perhaps precisely because comparison of mean responses is a core philosophy of almost all statistical testing in ecology. Yet changes in *variance* around the mean response can be at least as important as changes in mean response. Phenotypic variance—the product of genetic variation and phenotypic plasticity—is the raw material for differential selection, and thus lays at the heart of evolutionary responses to environmental variability and change. The importance of phenotypic variation both for bet-hedging and environmental buffering has been highlighted by many authors (see Jacobs and Podolsky, 2010, for a review in a marine context). Jacobs and Podolsky (2010) observe that although "studies of pattern in quantitative variation and its underlying causes have the potential to greatly expand our understanding of how selection works . . . quantitative comparisons of variation within and between populations are surprisingly rare" (pp. 639–640, Jacobs and Podolsky, 2010). Here, we address these issues by investigating the effects of control, stable OA, and fluctuating OA, on mean and variance of multiple response variables in the barnacle *Balanus improvisus*.

In barnacles, OA has been shown to decrease growth rate (*Elminius modestus*, Findlay *et al.*, 2010b), weaken shells (*Amphibalanus amphitrite*, McDonald *et al.*, 2009b; and *B. improvisus*, Pansch *et al.*, 2014), influence range-shifts (*Semibalanus balanoides*, Findlay *et al.*, 2010a, 2010c), and have no—or small—effects on larval development, survival, and settlement (several species; McDonald *et al.*, 2009a; Pansch *et al.*, 2012, 2013). Notably, the possibility that surplus energy can offset the negative effects of OA (Melzner *et al.*, 2011) has also been demonstrated for barnacles (Pansch *et al.*, 2014).

Balanus improvisus (Darwin) is eurythermal, euryhaline, and one of the most widespread barnacle species in the world (Foster, 1970; de Rivera *et al.*, 2011; Galil *et al.*, 2011). The species was introduced to Scandinavian waters around the mid-19th century (Blom, 1965; Foster, 1987) and is today found from 34 PSU in the Skagerrak down to 2 PSU in the Gulf of Bothnia (Leppakoski and Olenin, 2000). The population we studied, from Tjärnö on the Swedish west coast, typically experiences a salinity of 25 PSU and a maximum diurnal pH variation of ∼0.3 units (Supplementary Figure S1). We used *B. improvisus* from this location to investigate the effects of medium-term (3 month) stable and fluctuating OA on a variety of traits, including growth, survival, mineral composition of the shell, and shell strength.

Material and methods

Experimental design

The experimental system comprised three header tanks (50 l each) with a constant flow-through of natural surface seawater (temperature = 19°C ± 1; salinity = 26 ± 6; mean ± s.d.). Natural diurnal, and longer term, pH fluctuations in inflowing seawater were minimized by aerating the header tanks to pH ≈ 8.1 (≈380 ppm CO_2, Table 1) before water treatment. Treatments comprised constant ambient "control" (target pCO_2 = 380 ppm), "stable OA" (target pCO_2 = 970 ppm), and "fluctuating OA" [target pCO_2 = 1600 ppm at night (18.00–06.00 h) and 610 ppm during the day (06.00–18.00 h)]. The stable OA treatment reproduced projections for the year 2100 (Orr *et al.*, 2005; IPCC, 2014) and standard practice in most OA experiments, whereas the fluctuating OA treatment was designed to mimic natural diurnal fluctuations in pH observed in the field (plus or minus ∼0.2 pH units, Supplementary Figure S1) superimposed on the end of the century projections. Thus, by comparing results in "control" with "stable OA", we would mimic "typical" OA experiments, and by comparing "stable OA" with "fluctuating OA", we could determine the additional effects of fluctuating pH. Treatment levels were controlled with NBS-calibrated pH-computers (Aqua Medic GmbH, Germany) with a precision of ±0.01 pH units (≈12 ppm CO_2). Fluctuating treatments were obtained by using two such control

Table 1. Average pH, partial pressure of CO_2 (pCO_2), salinity, temperature, total alkalinity (A_T), dissolved inorganic carbon, and saturation state for calcite and aragonite (mean ± s.d.) in the control, stable OA, and fluctuating OA treatments during the 12-week experiment.

	pH_{NBS}	pCO_2 (μatm)	A_T (μmol kg^{-1})	Salinity (PSU)	T (°C)	C_T (μmol kg^{-1})	Ω_{Ca}	Ω_{Ar}
Control	8.10 ± 0.02	380	1567 ± 125	26 ± 4	19 ± 1	1498 ± 105	2.3 ± 0.5	1.4 ± 0.3
Stable OA	7.70 ± 0.03	970	1567 ± 125	26 ± 4	19 ± 1	1488 ± 92	0.9 ± 0.2	0.6 ± 0.1
Fluctuating OA								
Day	7.90 ± 0.02	625 ± 1	1567 ± 125	26 ± 4	19 ± 1	1499 ± 100	1.5 ± 0.3	0.9 ± 0.2
Night	7.50 ± 0.03	1665 ± 20	1567 ± 125	26 ± 4	19 ± 1	1586 ± 113	0.6 ± 0.1	0.4 ± 0.1

Salinity, temperature, and pCO_2 were measured or manipulated directly, A_T was estimated from salinity (see the Material and methods section), all other parameters were estimated with CO_2sys, using constants from Hansson and Merbach refit by Dickson and Millero (1987), Dickson (1990b), and Ho *et al.* (2006) as given by (Lewis and Wallace, 1998).

units with different set-points, the lower of which only operated between 18.00 and 06.00 h. pH_{NBS} set-points equivalent to desired pCO_2 levels in the treatments were determined by measuring pH_{NBS} in seawater equilibrated for 24 h with custom mixed gases at 380 or 970 μatmCO_2 (AGA, Sweden AB). To control for possible fluctuations in alkalinity (which would change the pH set-points required to achieve an equivalent pCO_2), calibrations were repeated every 2 weeks and the pH-computers adjusted accordingly. Total alkalinity was estimated from salinity using long-term salinity:alkalinity relationships for this location (SMHI, 2011; $r = 0.94$). Uncertainties arising from estimating alkalinity using this relationship were equivalent to ± 0.006 pH_{NBS} and 0.08 Ω_{Ar} (data for 99% CI around mean alkalinity estimated at 26 PSU, propagated through CO_2sys; Lewis and Wallace, 1998). Salinity (and hence alkalinity) fluctuations throughout the experimental period were relatively small (Table 1).

For each treatment, seven microcosms (5 l volume) were supplied with water from the respective header tank at 5 l h^{-1}. All tanks and microcosms were spatially interspersed to remove any artefacts arising from room effects, and all equipment was new to obviate any effects of prior history. Water and CO_2 flow rates into the header tanks, and microcosms, were manipulated, such that in the fluctuating acidification treatment, the transition from high to low pH in the microcosms took ~1 h. pH in each microcosm was monitored daily (NBS-calibrated Beckman Coulter pHi 460), and remained broadly consistent with target pCO_2s throughout the experiment (Table 1). Seawater salinity and temperature were recorded every 2–3 d using a YSI-30 conductivity meter. Carbonate system parameters were back-calculated from pH_{NBS} and A_T using the software CO_2sys using constants from Hansson and Merbach refitted by Dickson and Millero (1987), Dickson (1990b), and Ho *et al.* (2006) (Lewis and Wallace, 1998).

Balanus improvisus cyprid larvae were obtained from an established laboratory culture system (Berntsson *et al.*, 2000). Three separate collections of cyprid larvae were obtained from the barnacle culture that contained over 1000 adult broodstock. Collections (hereafter referred to as "batches") were made every third day within 1 week, and therefore, most likely represent output from different parents within the broodstock (mean frequency of larval release from individual *B. improvisus* is 5.81 ± 0.34 d, mean ± s.d.; JNH, pers. obs.). For each batch, cyprids were allowed to settle on acrylic panels in "control" seawater. After 48 h, panels bearing newly settled barnacles were distributed haphazardly among microcosms, so that each microcosm contained one panel from each larval batch (3 panels total). The mean barnacle density at the start of the experiment was 6.5 ± 2.9 per panel (mean ± s.d.). Panels were treated as replicates ($n = 7$ per batch). The experiment ran for 12 weeks (October–December 2010) under a 12:12 h light:dark cycle that coincided with the pH fluctuations (high pH during the day). In addition to natural food supplied from the inflowing seawater, every second day, barnacles were fed with a mixed microalgal diet at a final concentration of 20 000 cells ml^{-1} (Shellfish Diet 1800®). After 4 weeks, the barnacles had grown sufficiently to be able to capture and consume zooplankton, and therefore, their diet was supplemented with newly hatched *Artemia* nauplii.

Growth and survival

Barnacle growth and survival were recorded on each settlement panel every 2 weeks. Panels were temporarily removed from microcosms, cleaned gently and photographed in a standardized frame using an Olympus E-30 digital camera and 50 mm macro objective. A calibration scale was included in each image. Shell diameter (length and width) of all barnacles was measured from digital images using ImageJ (Abramoff *et al.*, 2004). Before photographing, any dead individuals were recorded and removed. Size was calculated only for barnacles that survived the entire experimental period (=3.95 ± 0.38 barnacles per panel, mean ± s.e.). Barnacle size throughout the experimental period was fitted against a Gompertz growth curve (Gompertz, 1825):

$$Y(t) = ae^{b\exp(ct)}$$

where Y is the diameter of the barnacle shell at time t, a the maximum diameter of the shell (asymptote), b represents the lag phase during early growth, and c is the maximum growth rate. Fitting this growth model facilitated a detailed understanding of the effects of the treatments on different components of growth (Wrange *et al.*, 2014).

Shell mineralogy

At the end of the experiment, shells from 14 individuals were haphazardly selected from each treatment. Mineral composition was determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Agilent 7500) calibrated with ICP multi-element standard solution (Merck, VI CertiPUR®). Samples comprised 10 mg of shell dissolved in 5% HNO_3, diluted ~50 000 times, with an internal standard containing 800 ppb indium. Drift and data quality were controlled using carbonate standards (Jls-1 and Jdo-1%). Accuracy was >97% and precision was >98.5% for both calcium and magnesium.

Shell strength

At the end of the experiment, shell strength was assessed in 16 individuals of similar size (shell length 5.7–6.7 mm), four from each treatment/batch. Crushing resistance of the rostral shell plate was measured using a Mark-10 force-gauge (ESM 3000). Shell plates were crushed with a blunt needle fixed to the force sensor (SSM10, accuracy ± 0.15 N) perpendicular to the centre of the plate at 0.25 m min^{-1}. Accumulated force required to crush the plate (to failure) was recorded.

Statistical analyses

Responses to pH treatments were visualized using log response ratios (LnRR; Nakagawa and Cuthill, 2007; Koricheva *et al.*, 2013). Survival, shell mineralogy, and shell strength were also analysed using two-way mixed-model ANOVA to test for effects of treatment (fixed) and batch (random). Growth data over time were compared between treatments and batches using repeated-measures ANOVA. Before statistical analysis, all data were tested for homogeneity of variance using Levene's test and for normality using Q–Q plots. Where necessary, data were transformed to meet assumptions (Quinn and Keough, 2002). Statistical analyses were performed using SPSS (IBM SPSS Statistics v. 20).

Results

Water chemistry

pH was relatively stable throughout the experimental period (Table 1) with a transition time from low pH to high pH (or *vice versa*) in the fluctuating treatment of ~60 min. Small fluctuations in carbonate chemistry parameters arose due to salinity variation in the natural seawater flowing through the system (Table 1). "Controls" were saturated with respect to calcite and aragonite

over the whole experimental period, whereas "stable OA" treatments were near saturation for calcite and under-saturated for aragonite, and "fluctuating OA" treatments were under-saturated for both calcite and aragonite at the lowest (night-time) pH levels (pH = 7.50; Table 1).

Growth and survival

By the end of the experiment (12 weeks after settlement), barnacles had reached adult size (shell diameter ~6.4 mm; Figure 1). In all treatments, maximum growth rate occurred after 4–6 weeks, at which time most individuals doubled in size within 2 weeks (Figure 1). Despite apparent trends for slower growth in stable OA and more rapid growth in fluctuating OA (Figure 1), log response ratios (LnRRs) showed that mean effect sizes were either very small or zero for all growth parameters (Figure 2). This result was corroborated by formal statistical analysis, which showed no significant effects of treatment on growth rate (Supplementary Table S2). Surprisingly, however, 95% confidence intervals (CIs) for LnRRs under fluctuating OA were almost 20-fold greater than for those under stable OA (Figure 2). This indicates that *variation* in growth responses was far greater under fluctuating acidification. This pattern of markedly increased variance in responses to fluctuating acidification was also seen in shell chemistry metrics (see below).

Growth rate varied significantly between barnacle batches (batch × time interaction; $F = 11.3$, $p < 0.001$; Supplementary Table S2), indicating that parentage may influence growth (replicate batches were likely produced by different subsets of the barnacle broodstock; see the Material and methods section). Importantly, however, there was no significant effect of treatment on this interaction (batch × time × treatment, $F = 0.47$, $p = 0.98$, Supplementary Table S2).

Both stable and fluctuating OA had only small effects on mean barnacle survival (Figures 2 and 3). Survival differed between batches (Figure 2) such that some batches showed no influence of pH treatment, whereas there were significant negative effects of both stable and fluctuating acidification for others (95% CIs do not overlap zero, Figure 2). This result was supported by formal analysis that showed a marginally significant effect of batch ($F = 3.09$, $p = 0.054$, Supplementary Table S3), and no significant effect of pH or pH × batch interaction (Supplementary Table S3). In contrast to the growth data, there were no apparent differences in the 95% CIs LnRRs for survival in stable and fluctuating treatments (Figure 2).

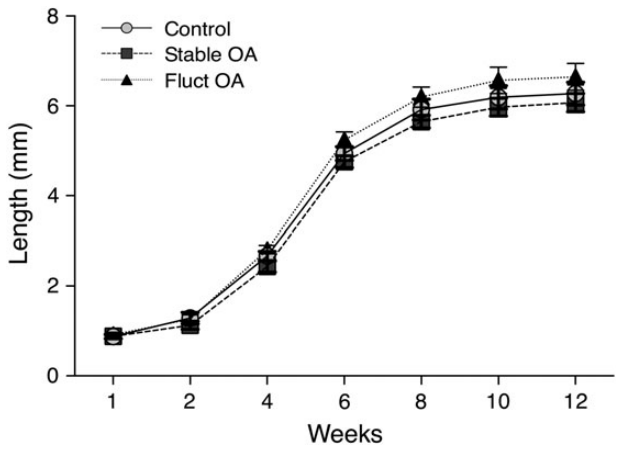

Figure 1. Mean shell diameter (± s.e.) of barnacles during the experimental period under different pH treatments (means of 3 batches per treatment, $n = 7$ replicate panels per batch).

Shell mineralogy

As for the growth data, the mean effect sizes for shell mineralogy in the different treatments were small, or zero (Figure 2), yet there were large differences in the magnitudes of the 95% CIs around these effect sizes for the different treatments (Figure 2). Once again, this indicates that variation in shell mineralogy was far greater under fluctuating acidification than under stable OA. ICP-MS indicated that both Ca and Mg content declined with acidification (control > stable OA > fluctuating OA; Supplementary Table S4), but these differences were small, and formal statistical analysis indicated no significant effects of treatment on either Ca content or Ca:Mg ratio (Supplementary Table S5). As was seen earlier for growth and survival, there was a significant effect of batch on Ca:Mg ratio (ANOVA, $F = 3.55$, $p = 0.038$; Supplementary Table S5).

Shell strength

Both stable and fluctuating acidification had a negative impact on the force (N) needed to penetrate the rostral shell plate of barnacles (Figure 4). The mean effect sizes for both OA treatments were similar

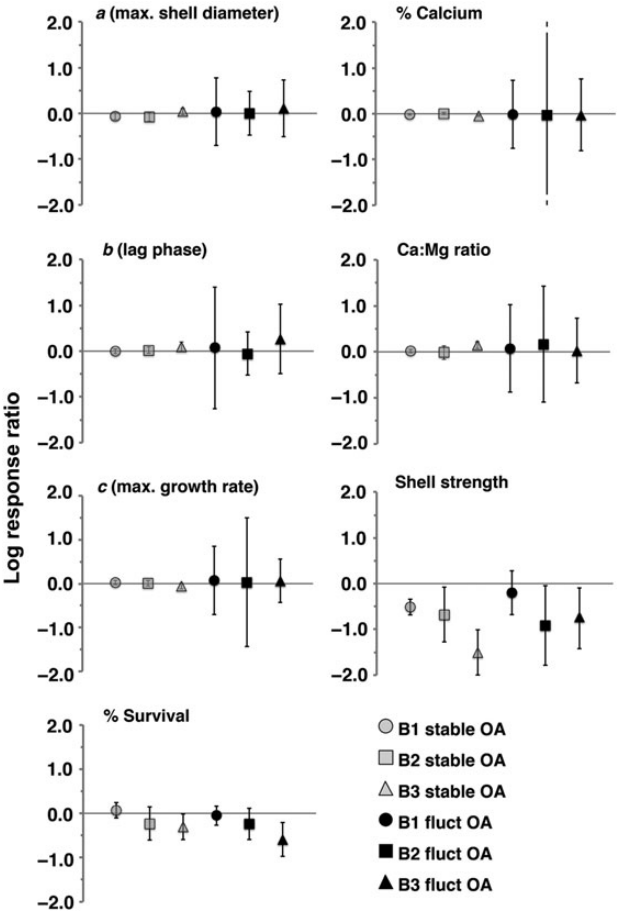

Figure 2. Log response ratios (mean ± 95% CI) for the effects of acidification (relative to controls) for Gompertz growth model parameters (*a*, *b*, and *c*; see text for details), % survival, % calcium content of shell, Ca:Mg ratio, and shell strength. Data are for "stable OA" (grey) and "fluctuating OA" treatments for each of the three batches (B1, B2, and B3; $n = 7$ replicate panels per batch).

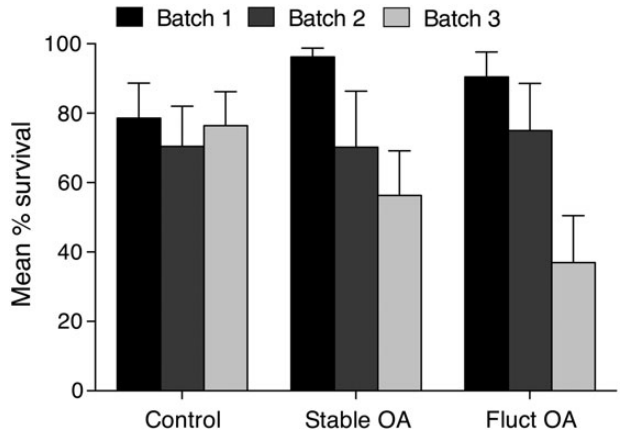

Figure 3. Percentage survival (mean ± s.e.) of barnacles for each pH treatment and batch over the experimental period ($n = 7$).

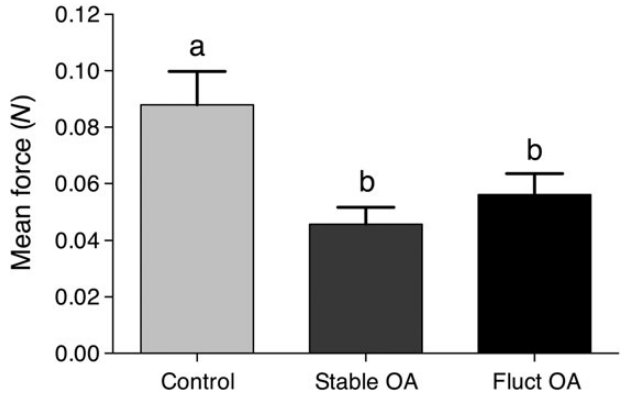

Figure 4. Force (mean ± s.e.) needed to penetrate the rostral shell plate of barnacles reared in three pH treatments, 12 weeks after cyprid settlement ($n = 14$). Letters indicate significantly different groups (Tukey's test, $p \leq 0.05$).

within batches and markedly negative, with 95% CIs that did not overlap zero for all but one of the batches (Figure 2; see also Supplementary Table S6). The variation in responses of different batches apparent in Figure 2 was not, however, reflected in formal statistical analysis (Supplementary Table S6). Shell strength was independent of shell thickness ($r = 0.183$, $p = 0.178$).

Discussion

Our finding that fluctuating OA had no effect on mean response, but caused an ~20-fold increase in *variance* of response (compared with stable OA; Figure 2) is unprecedented. The few published studies that have tested the effects of fluctuating OA (hereafter termed "fluctuating acidification") have found significant effects on mean responses (Alenius and Munguia, 2012; Dufault *et al.*, 2012; Cornwall *et al.*, 2013; Johnson *et al.*, 2014; Ou *et al.*, 2015), but none of these studies found the marked effects of fluctuating acidification on variance around mean responses that we report here.

In our experiments, greater variance in response ratios for fluctuating acidification was caused by some replicates showing markedly more positive responses, and some markedly more negative responses, than was seen under stable acidification. Without further investigation, the mechanisms by which this variation arose remain unclear; however, it is evident from Figure 2 that the growth and shell mineralogy of some individuals responded positively, and strongly, to fluctuating acidification (upper 95% CIs for fluctuating OA were typically ≈1.0 $\log_e$ unit, equivalent to nearly 3× greater growth, Ca content, or Ca:Mg ratio than was observed in the controls). Increased variance in mean response has previously been reported as indicative of "winners and losers" resulting from climate change (Loya *et al.*, 2001; Schlegel *et al.*, 2012). A key issue in this context is whether or not this variance reflects heritable genetic differences among barnacles or is rather the result of adult plasticity, acclimation, or environmental effects (Somero, 2010; Schlegel *et al.*, 2012). As our barnacle broodstock were collected simultaneously as newly settled juveniles from one location (Tjärnö, western Sweden), and raised together in the laboratory for 5 months at constant temperature and salinity before use in our experiments, we suggest that it is unlikely that the observed differences among replicates were due to parental acclimation to different environments and subsequent trans-generational inheritance of that plasticity (e.g. Miller *et al.*, 2012). Rather, it is far more plausible that the different responses we observed among replicates were the result of genetic differences among those replicates. It follows, therefore, that natural pH-fluctuations in present day—as well as acidified future—oceans may select for genetic variants that are not only tolerant of, but actually benefit from, future acidification. This is a biologically important finding that warrants further research.

In contrast to the results for growth and shell mineralogy, variances of response ratios for shell strength and survival did not differ between fluctuating and stable acidification treatments (Figure 2). This finding suggests a basic difference in the mechanisms by which growth and shell composition on the one hand, and survival and shell strength on the other, respond to fluctuating acidification. The mean shell strength was significantly, and negatively, influenced by acidification ($p = 0.013$, Supplementary Table S6), but the effects of fluctuating and stable acidification treatments did not differ (mean force to penetrate rostral plates was 0.089, 0.047, and 0.055 N for control, stable acidification, and fluctuating acidification treatments, respectively). Note that there were no predators in our microcosms and therefore no clear candidate mechanism for a causal link between shell strength and individual survival.

Previous studies have found that barnacles are generally tolerant of (stable) acidification. OA has been reported to only slightly decrease growth rates (*E. modestus*, Findlay *et al.*, 2010b), and have no—or small—effects on larval development rate, settlement, survival, and calcification rates (several species, McDonald *et al.*, 2009a; Pansch *et al.*, 2012, 2013). However, in keeping with our findings, other studies have reported that acidification resulted in weakened shells (*A. amphitrite*, McDonald *et al.*, 2009c; and *B. improvisus,* Pansch *et al.*, 2014), although McDonald *et al.* (2009b) also observed increased calcification in some parts of the shell (basal plate) under acidification. Acidification-induced changes in hardness of shells, such as those seen here (Figure 2, Supplementary Table S3), could have major implications for survival of barnacles. A weaker shell would leave them more susceptible to mortality by abrasion from ice and/or predation (e.g. from shore crabs, Buchsbaum, 2002).

Pansch *et al.* (2014) concluded that food limitation could enhance the negative effects of acidification on barnacles, but also mediate negative effects of OA when food was plentiful. We did not measure food supply in our study; however, we assumed this not to be limiting, as animals were fed every second day as well as receiving natural food through the flow-through seawater system.

The statistical strength of our experiments was aided by the use of three replicate batches of larvae from the same barnacle broodstock. Reproductively active pairs of *B. improvisus* in our cultures released batches of larvae on average every 5.81 d (range 5–62 d; JNH, pers. obs.), and our broodstock comprised over 1000 active individuals. Thus, by using three batches of larvae collected within 1 week, we maximized the likelihood that each batch derived from different parents and therefore represented a different genetic sample from the broodstock population (although we cannot exclude the possibility that a small number of parents may have contributed to both batch 1 and 3). Consistent with this aim, mean responses to shell strength and survival (the only two variables that showed a mean effect of treatment; Figure 2) were always greatest in batch 3, and weakest in batch 1 (Figure 2). As we noted earlier in our discussion of the consequences of increased variance in response ratios, this consistent difference between batches also constitutes evidence in support of the notion of "winners and losers"—although given the near-zero response ratios for batch 1 in both shell strength and survival (Figure 2), the distinction is perhaps better defined as being between "nonchalants and losers".

The differences we observed in the responses of different batches also emphasize the oft-repeated and oft-overlooked importance of using different genotypes (or populations) as replicates in experiments. Limiting the spatial and temporal extent of our samples—the "sampling universe"—limits the spatio-temporal extent of our conclusions, and can meaningfully constrain the validity and usefulness of our results (Havenhand *et al.*, 2010). Given this, we emphasize that the results we report here are for one population of *B. improvisus* from western Sweden, and that other populations of this same species—or indeed other species of barnacle—may well respond differently. Available evidence suggests that this is perhaps not likely in this case as phenotypic and genotypic differences among *B. improvisus* populations from Scandinavia and northern Germany are not great (Nasrolahi *et al.*, 2012; Wrange *et al.*, 2014; but see Pansch *et al.*, 2013), and consequently, we might expect other populations of this species to respond similarly to the patterns seen here. However, we stress the importance of testing for the effects of acidification—and other stressors—on multiple populations to ascertain the degree to which responses obtained from limited sampling universes can be generalized to whole species (e.g. Langer *et al.*, 2009; Walther *et al.*, 2010, 2011).

Although the field of OA research is still relatively new (Riebesell *et al.*, 2010), substantial advances have been made since Raven *et al.* (2005) brought this issue to a wider audience. The importance of fluctuating ocean pH is beginning to be recognized, as evidenced by the 200+ citations of Hofmann *et al.* (2011) in the 4 years since its publication. Yet few studies (to our knowledge, seven, including this one—see references above) have investigated experimentally the effects of simulated diurnal fluctuations in pH on organisms. Our finding that fluctuating pH has near-zero effects on mean responses of some, but not all, of the response variables we measured is perhaps not surprising: there is clear reporting bias against non-significant effects of OA (although this Special Issue takes an important step to address this bias). However, the increase in *variance* in responses that we observed under fluctuating acidification suggests that our previous focus on the effects of stable acidification has led us to miss environmentally and evolutionarily important responses of organisms. The challenge of investigating the combined effects of multiple simultaneously fluctuating environmental variables remains to be answered, but the results presented here and elsewhere (Alenius and Munguia, 2012; Dufault *et al.*, 2012; Cornwall *et al.*, 2013; Comeau *et al.*, 2014; Johnson *et al.*, 2014; Ou *et al.*, 2015) indicate that this is an important next step.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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