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Impacts of ocean acidification on survival, growth, and swimming behaviours differ between larval urchins and brittlestars

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Ocean acidification (OA) is widely recognized as an increasing threat to marine ecosystems. Many marine invertebrates have dual-phase life cycles in which planktonic larvae connect and sustain otherwise disconnected benthic adult populations. Many planktonic larvae are particularly sensitive to environmental stresses including OA. Here, we compared the developmental dynamics, survivorship, and swimming behaviours of plutei of two ecologically important echinoderm species that naturally experience variability in ambient pH: the purple urchin *Strongylocentrotus purpuratus* and the infaunal brittlestar *Amphiura filiformis*. Sensitivity to decreased pH differed between these two species and between maternal lineages. Larvae of both species experienced increased mortality and reduced growth rate under low pH conditions. However, larval brittlestars appeared more sensitive and experienced over 80% mortality after 7-d exposure to pH 7.7. Larval urchins from one maternal lineage underwent highly synchronized budding (release of blastula-like particles) at low pH. Observed budding temporarily increased numerical density and reduced individual size, leading to differences in growth and mortality rates between the two half-sibling groups and another population. Swimming speeds of larval brittlestars were reduced in decreased pH. In contrast, acidification had either no effect or positive effect on swimming speeds of larval urchins. The observed differences between species may be a reflection of pre-exposure in their natural habitats: larval brittlestars experience a relatively stable *in situ* pH environment, whereas larval urchins are occasionally exposed to low pH in upwelling regions. Urchins may therefore exhibit short-term compensatory responses such as budding and increased swimming speed. Natural selection could act upon the significant variations we observed between maternal lineages, resulting in more resilient populations confronting chronic exposure to OA.

Keywords: *Amphiura filiformis*, echinopluteus, global climate change, *Strongylocentrotus purpuratus*, video motion analysis.

Introduction

Dissolution of anthropogenic carbon dioxide (CO_2) from the atmosphere into the ocean has altered its carbonate chemistry and led to reduced pH, a process known as ocean acidification (OA). The average surface ocean pCO_2 level is predicted to rise to 1000 μatm by 2100, causing pH to drop from 8.1 to 7.7 units (Caldeira and Wickett, 2003; Zeebe, 2012). A growing body of evidence suggests that these changes can significantly affect survival, growth, behaviour, and physiology of diverse marine organisms (Dupont and Thorndyke, 2013; Dupont and Pörtner, 2013).

For benthic organisms, the planktonic larval stage is generally considered the most vulnerable part of the dual-phase life history (Kurihara, 2008; Byrne, 2011; Aze *et al.*, 2014). However, vulnerability differs between species. Larvae of some species are sensitive to OA and experience high mortality (e.g. Dupont *et al.*, 2008), whereas others experience sublethal effects (see Dupont *et al.*, 2010a, for review) or even positive effects (e.g. Dupont *et al.*, 2010b; Kroeker *et al.*, 2013). These impacts include increased metabolic costs (e.g. Stumpp *et al.*, 2011b, 2012; Matson *et al.*, 2012), altered feeding physiology (e.g. Stumpp *et al.*, 2013; Challener *et al.*, 2014), delays

in growth and development (e.g. Brennand *et al.*, 2010; Martin *et al.*, 2011; Dorey *et al.*, 2013), and changes in gene expression patterns (e.g. O'Donnell *et al.*, 2009; Stumpp *et al.*, 2011a; Evans and Watson-Wynn, 2014). It is assumed that failure to perform key physiological functions (feeding, growth, acid-base regulation, calcification, etc.) can translate into reduced fitness. For example, a delay in development can translate into significant mortality due to predation during a prolonged planktonic period (Morgan, 1995; Dorey *et al.*, 2013). Aside from physiological vulnerability to environmental stresses, another critical aspect of larval ecology is their key role as vehicles for dispersal and genetic exchange. This dispersive role is significantly affected by larval swimming behaviours: individual movements affect their vertical distribution, and hence, the environmental conditions encountered (Metaxas and Saunders, 2009). The impact of OA on larval swimming and dispersal potential has received surprisingly little attention (but see Chan *et al.*, 2011). However, swimming and other OA impacts on larval stages are likely to affect both evolutionary and demographic dynamics of vulnerable species.

Echinoderms play important roles in coastal ecosystems and are ideal candidates for studying ecologically significant elements of organismal performance, such as swimming, under acidification stress. Plutei larvae of echinoderms have long ciliated projections (arms) attached to a pyramid-shaped larval body. Larvae add pairs of arms throughout larval growth and development. These arms are supported by calcified structures and are used for both feeding and swimming (Strathmann, 1975). Modelling and experimental studies have shown that slight changes in larval morphology significantly impact larval feeding and swimming and suggest trade-offs between the two functions (Grünbaum and Strathmann, 2003; Strathmann and Grunbaum, 2006; Clay and Grünbaum, 2011). When reared under reduced pH conditions, the larval sand dollar, *Dendraster excentricus*, showed morphological changes, including reduced stomach size, but showed no reduction in swimming speeds (Chan *et al.*, 2011). This observation suggests that OA could alter trade-offs in larval morphology between swimming and feeding.

Many echinoderms inhabit coastal areas where they naturally experience exposure to reduced pH, e.g. intertidal habitats, upwelling zones, and sediments (Cai and Reimers, 1993; Dai *et al.*, 2009; Yu *et al.*, 2011; Hu *et al.*, 2014). These echinoderms are good models to investigate possible adaptations to cope with reduced pH (Hoffmann and Sgrò, 2011; Kelly *et al.*, 2013; Pespeni *et al.*, 2013; Sunday *et al.*, 2014). In this study, we compared two echinoderm species that naturally experience low pH conditions as adults, the purple urchin *Strongylocentrotus purpuratus* and the infaunal brittlestar *Amphiura filiformis*.

Strongylocentrotus purpuratus is an abundant, long-lived species and key component of the intertidal and subtidal zone of the Eastern Pacific coast, with a distribution range from Mexico to Alaska (Biermann *et al.*, 2003). Its distribution includes upwelling areas in which both adult and larval stages can experience low pH levels (Yu *et al.*, 2011). Larval *S. purpuratus* were the focus of several previous OA studies (see Dupont and Thorndyke, 2013, for review). When reared under low pH conditions, no differences were observed in the first cell division (Place and Smith, 2012). Larval survivorship was not reduced by low pH (Matson *et al.*, 2012); however, larvae exposed to reduced pH had smaller size (Stumpp *et al.*, 2011b; Yu *et al.*, 2011) and slower growth rate (Stumpp *et al.*, 2011b; Dorey *et al.*, 2013). Larval respiration rates increased at low pH, whereas feeding rate remained unaffected (Stumpp *et al.*, 2013), suggesting a shift in energy budget with less energy available for somatic growth (Stumpp *et al.*, 2011b; Matson *et al.*, 2012). With a fully sequenced genome, the purple urchin was one of the first species investigated using genomics tools. Impacts of OA on embryos and larval transcriptomics were assessed using both microarrays (Todgham and Hofmann, 2009) and qPCR (Stumpp *et al.*, 2011a; Hammond and Hofmann, 2012). However, only subtle differences in gene expression were observed. *Strongylocentrotus purpuratus* presented apparently high adaptation potential to OA, with differential changes in allele frequencies in larvae grown under pH 7.0, suggesting allele-specific survival (Pespeni *et al.*, 2013).

Amphiura filiformis is an important species in many polar and temperate marine benthic habitats, with densities reaching 3500 ind. m^{-2} (Rosenberg *et al.*, 1997). It lives in semi-permanent sediment burrows. Within these burrows, adults often experience hypoxic and reduced pH conditions (Hu *et al.*, 2014). However, spawning occurs in summer when the pH level is relatively high and larval stages never experience *in situ* pH lower than pH 7.9 (Dorey *et al.*, 2013). When exposed to OA scenarios, adult brittlestars experienced only sublethal effects, e.g. muscle wastage (Wood *et al.*, 2008; Hu *et al.*, 2014). No information is available on the impacts of OA on the larval stages of *A. filiformis*. However, a study on another brittlestar found in similar habitats, *Ophiothrix fragilis*, observed extreme larval sensitivity of larvae to OA: larvae suffered 100% mortality within a few days of exposure to low pH (Dupont *et al.*, 2008).

Here, we focus on the vulnerable larval life-history stage to investigate the impacts of the reduced pH level on survival, growth, and swimming behaviours of these two echinoderms. Due to differences in their reproductive timing, larval purple urchins, *S. purpuratus*, experience variable pH but larval brittlestars, *A. filiformis*, develop in a relatively stable pH environment. Interspecific differences in responses between these echinoderms to stresses like OA could be interpreted as indicators of potentially altered ecological interactions that may promote shifts in abundance or range relative to other species comprising their marine communities. Intraspecific differences in stress responses could be interpreted as variability subject to natural selection, and hence as indicators of potential for rapid adaptation to changing environmental conditions. Comparative studies of ecologically significant organismal performance are therefore essential for understanding future population and community dynamics.

Material and methods

Animal collection and spawning

Adults of the sea urchin *S. purpuratus* were collected from 25 to 30-m depth in San Diego Bay, CA, USA, and transferred to the Sven Lovén Centre for Marine Sciences—Kristineberg (Fiskebäckskil, Sweden) in June 2011. They were maintained in a flow-through system at 11°C, salinity 32, pH_T 8.0, and fed *ad libitum* on a diet of *Ulva lactuca*. Spawning was induced by injecting 0.5–1 ml of 0.5 M KCl into the coelomic cavity (Strathmann, 1987). Eggs from females were collected in filtered seawater (FSW, 0.45 μm) at 11°C, salinity 32, pH_T 8.0, and sperm were collected dry and kept on ice until use. Sperm stock solution in FSW was added to the egg suspension to a final concentration of ∼1000 sperm ml^{-1}. After 15 min and confirmation that there was >95% fertilization success, fertilized eggs were rinsed with FSW.

Adults of the brittlestar *A. filiformis* were collected at 25–40-m depth using a Petersen mud grab in the Gullmarsfjord near the

Sven Lovén Centre for Marine Sciences—Kristineberg (Fiskebäckskil, Sweden) between July and August 2011. Individuals were immediately sampled from the sediment cores by gentle rinsing then maintained in natural flowing seawater at 11°C, salinity 32, and pH_T 8.0. Individuals with ripe gonads (white for testes and orange for ovaries) which were clearly visible through the extended wall of bursae were used for fertilization. Three males and 15 females were heat-shocked at 26°C for 15 min then kept in 1 l of FSW in the dark at 11°C until the release of sperm that induces the female to spawn (Dupont *et al.*, 2009). Fertilized eggs were rinsed in FSW after confirming fertilization success.

Larval rearing and carbonate chemistry

Two-cell stage embryos of both species were transferred at the density of ~5 ind. ml^{-1} to aerated 5 l culturing vessels containing FSW. Three experiments were started: experiment one with *A. filiformis* from mixed gametes from 3 males and 15 females, experiment two with *S. purpuratus* gametes from two different females fertilized with the same male (maternal half-siblings, hereafter females 1 and 2), and experiment three with a common garden culture with gametes two male and two females of *S. purpuratus* (hereafter mixed population). Duplicate or triplicate cultures were set up for each pH treatment. Larvae were reared at 14°C and salinity 32 in a 12:12 light–dark cycle and were fed *Rhodomonas* sp. at 150 $\mu gC\ l^{-1}$ (~4000–5000 cells ml^{-1}) starting from 5 d post-fertilization. The carbon content of the algae was estimated using biovolume measurements, quantified as equivalent spherical diameter with an electronic particle analyser (Elzone 5380, Micrometrics, Aachen, Germany; Mullin, 1966). Complete water changes were performed every 3 d.

pH in each culturing vessel was maintained using a computerized feedback system (AquaMedic) that regulated pH by addition of pure gaseous CO_2 directly into the seawater (± 0.02 pH units). *Strongylocentrotus purpuratus* larvae were reared at three nominal pH levels: 8.0, 7.7, and 7.3. Based on a pilot study, *A. filiformis* larvae were reared only in pH 8.1 and 7.7, due to high larval mortality under more acidified conditions (Dupont *et al.*, pers. obs.) Our experimental scenarios correspond to (i) average pH experienced today (pH 8.0); (ii) average pH in 2100 and extreme of present natural variability (pH 7.7); and (iii) extreme pH in 2100 outside of present natural variability (pH 7.3; Dorey *et al.*, 2013). pH was measured every second day with a Metrohm 827 pH lab electrode adjusted for pH measurements on the total scale (pH_T) using TRIS (Tris–HCl) and AMP (2-aminopyridine/HCl) buffer solutions with a salinity of 32 (provided by Unité d'Océanographie Chimique, Université de Liège, Belgium). Alkalinity was measured weekly following Sarazin *et al.* (1999). Carbonate system speciation (pCO_2, Ω_{Ca}, and Ω_{Ar}) was calculated from pH_T and alkalinity using CO2SYS (Pierrot *et al.*, 2006) with dissociation constants from Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987).

Mortality, budding, and growth

Larval density (ind. ml^{-1}) was monitored daily for the duration of the experiments (14–26 d for *S. purpuratus*, 7 d for *A. filiformis*) by taking duplicate 10 ml subsamples from the culturing vessels. Individuals were immediately fixed with a drop of paraformaldehyde solution (4% PFA in FSW, buffered at pH 8.3), counted, and stored in 4% PFA solution at 4°C until further measurements. Visual evidence of budding—release of blastula-like particles (Chan *et al.*, 2013)—was recorded. Larval densities were normalized relative to initial densities, then compared between pH treatments using ANCOVA with age as a covariate.

To measure larval growth, photographs of ten larvae per replicate at several time points and body length (TL, in μm) were measured with ImageJ (Figure 1, Abràmoff *et al.*, 2004). The larval growth rate [ln(μm) d^{-1}] was calculated as the coefficient of the significant logarithmic regressions between TL and time (Stumpp *et al.*, 2011b). The effect of pH on TL was tested using ANCOVA with age as a covariate following a logarithmic transformation.

Video motion analysis

Larval behaviours were quantified using the video motion analysis methods detailed in Chan and Grünbaum (2010) and Chan *et al.* (2011). Briefly, ~100 individuals were randomly selected and gently injected into a 2.5 × 2.5 × 30-cm plexiglas chamber containing FSW equilibrated to the respective pH levels. Six chambers were submerged in a water bath maintained at 11°C. Two-minute long video clips were collected at 15 frames s^{-1} with two modified webcams (Logitech webcam Pro9000) equipped with 7.5 mm CCTV lens (Rainbow Tech. Inc., AL, USA). Cameras were mounted on a computer-controlled platform to capture video from two fields of view, 0–13 and 13–25 cm. Video clips were collected every 6 min for a total of 36 min. Two more sets of observations were made on the same group of larvae after gently mixing the chambers to redistribute individuals. Video clips were analysed with a customized version of Avidemux2.4 software to subtract background, to threshold for size and brightness, and to extract pixel coordinates. An in-house Matlab program, Tracker3D, was used to calibrate and to assemble individual tracks over time.

Using smoothing splines with different knot spacing, frame rate noise was removed to generate smoothed track and the overall direction of travel (axis) was differentiated from the oscillatory (helical) components of each individual trajectory. Weighted means of six swimming metrics were computed by sampling at 5 s time intervals and averaging across tracks intersecting each time point (see Chan and Grünbaum, 2010, for details). Three key swimming metrics presented here: gross speed (the averaged magnitude of the time derivative of the smoothed track), and net horizontal and vertical velocities (the displacement between the starting and ending point of the track divided by observation duration).

Effects of pH treatments were tested using ANCOVA with larval age or calculated TL as covariate. TL at a given day was calculated using the logarithmic regression for growth described above to test if changes in the growth rate measured by size alone could account for the differences in swimming behaviours. Larval swimming did not vary within the 36 min of observation or between repeated observations (data not shown); therefore, these factors were not included as random factors in the statistical analysis. *Post hoc* analyses with Bonferroni corrections were conducted.

All data were checked for distribution and homoscedasticity. All statistical analyses were conducted using PASW Statistics 13 (IBM).

Results

Seawater chemistry

Mean ± SD for seawater carbonate chemistry parameters measured and calculated for all species, treatments, and replicates are reported in Table 1. The pH treatment had a significant effect on all carbonate chemistry parameters (ANOVA 3, model: $F_{15,\ 106} > 30$, $p < 0.0001$, parameter: $p < 0.0001$), except on alkalinity, where there was no difference between species ($p > 0.05$), replicates ($p > 0.05$), or their interactions ($p > 0.05$). Mean pH_T were 8.04 ± 0.02, 7.69 ± 0.02, and 7.27 ± 0.03 for nominal pH 8.0, 7.7, and 7.3, respectively.

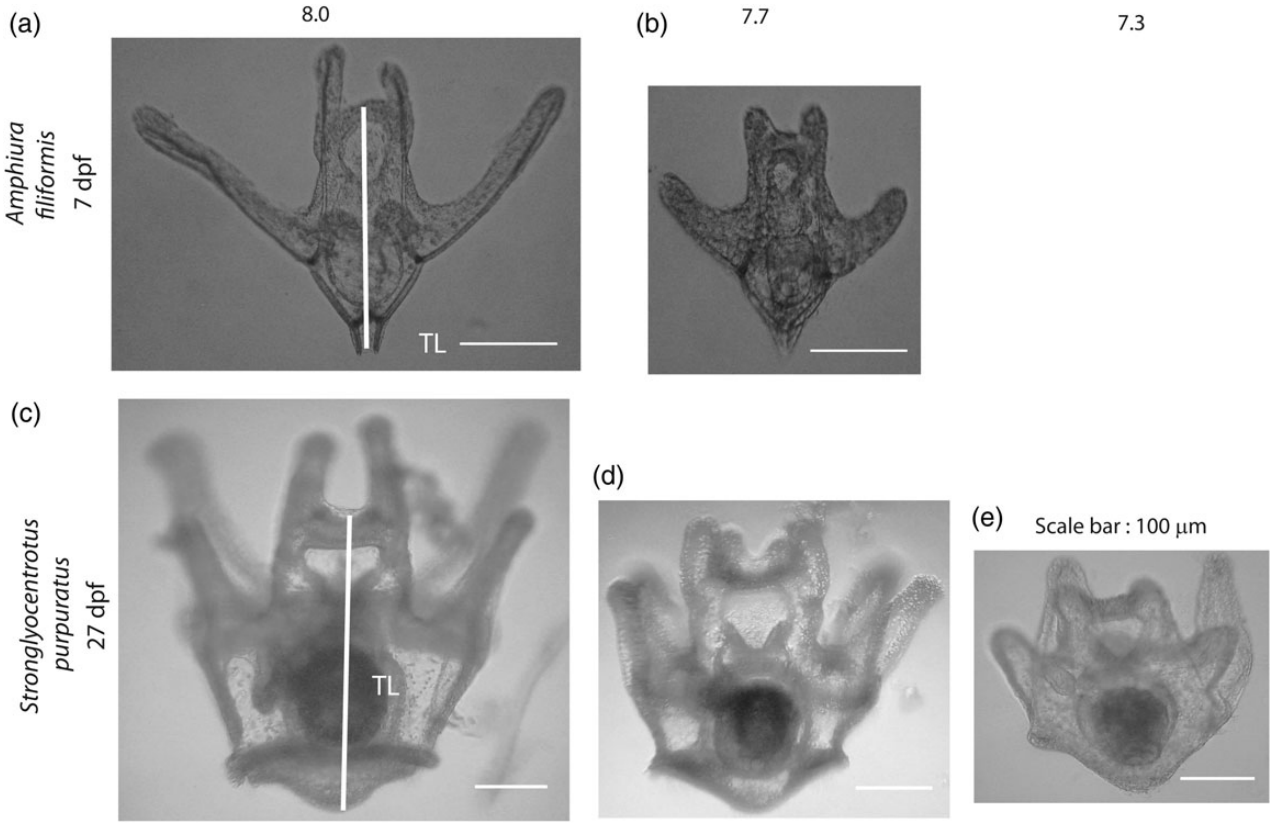

Figure 1. Micrographs of representative of 13 d post-fertilization (dpf) larvae of *A. filiformis* reared under pH 8.0 (a) and pH 7.7 (b); and 27 dpf larvae of *S. purpuratus* reared under pH 8.0 (c), pH 7.7 (d), and pH 7.3 (e). White bar corresponds to the measured total body length (TL). All scale bars are 100 µm.

Seawater was under-saturated regarding both calcite and aragonite in the lowest pH treatment.

Effects of decreased pH on growth

The larval growth rate [ln(µm) d^{-1}] was computed as the coefficient of logarithmic regression between total body length (µm) and larval age (d). All regressions for both *A. filiformis* and *S. purpuratus* were statistically significant ($p < 0.0001$, Supplementary Table S1).

For larval *A. filiformis*, both age and pH treatment had significant effect on growth rates, with larvae in decreased pH growing significantly slower (ANCOVA, age: $F_{1, 309} = 275.276$, $p < 0.0001$, pH: $F_{1, 309} = 36.254$, $p < 0.001$, Figure 2a).

Larval *S. purpuratus* from both maternal lineages (females 1 and 2) showed similar responses. Larvae reared under decreased pH had significantly slower growth (ANCOVA, female 1, age: $F_{1, 229} = 407.106$, $p < 0.001$; pH: $F_{2, 229} = 104.771$, $p < 0.001$; female 2, age: $F_{1, 226} = 312.531$, $p < 0.001$, pH: $F_{2, 226} = 134.317$, $p < 0.001$). *Post hoc* tests showed that observed growth rates in the three pH treatments were significantly different from each other (Figure 2b and c). Significant differences in growth rates were observed between the two females (ANCOVA, $F_{1, 456} = 8.156$, $p = 0.004$) but there were no significant female × pH interactions ($F_{2, 456} = 0.771$, $p = 0.463$). Larval growth rates were compared within each pH treatment. Larvae from female 1 had significantly higher growth rate at pH 7.7 compared with female 2 (ANCOVA, $F_{1, 147} = 11.564$, $p = 0.001$) but there were no significant differences between females in the other two tested pH treatments (ANCOVA, pH 8.1: $F_{1, 146} = 1.590$, $p = 0.209$; pH 7.3: $F_{1, 161} = 0.734$, $p = 0.393$).

Effects of decreased pH on mortality

Relative larval mortality rates (RMR) were calculated as the coefficient of the significant relationship between relative larval densities and either age (d^{-1}) or calculated total body length (using regressions in Supplementary Table S1; in μm^{-1}). All regressions for both species were statistically significant (Supplementary Table S2).

Larvae of *A. filiformis* raised in decreased pH experienced significantly greater mortality rate both expressed per age (RMR in d^{-1}, ANCOVA, age: $F_{1,34} = 54.375$, $p < 0.0001$, pH: $F_{1,34} = 12.93$, $p = 0.001$) and per total body length (RMR in μm^{-1}, ANCOVA, body length: $F_{1, 34} = 30.629$, $p < 0.0001$, pH: $F_{1, 34} = 20.428$, $p < 0.0001$).

pH treatment had significant effects on relative mortality rates of larval *S. purpuratus* of both female 1 (RMR in d^{-1}, ANCOVA, age: $F_{1, 135} = 70.071$, $p < 0.0001$, pH: $F_{2, 135} = 10.121$, $p < 0.0001$; RMR in μm^{-1}, ANCOVA, age: $F_{1, 135} = 41.259$, $p < 0.0001$, pH: $F_{2, 135} = 17.439$, $p < 0.0001$) and female 2 (RMR in d^{-1}, ANCOVA, age: $F_{1, 141} = 185.028$, $p < 0.0001$, pH: $F_{2, 141} = 19.354$, $p < 0.0001$; RMR in μm^{-1}, ANCOVA, age: $F_{1, 141} = 167.582$, $p < 0.0001$, pH: $F_{2, 141} = 63.121$, $p < 0.0001$). *Post hoc* tests showed two different patterns for the two females. For female 1, RMR of individuals reared under pH 7.7 was significantly higher than the two other treatments. For female 2, RMR of individuals reared at pH 7.3 was significantly higher than those reared at pH 8.0 (*Post hoc* Tukey's test, $p < 0.05$). When comparing the two females using an ANCOVA with pH as the fixed factor and age or size as the covariate, both female and the interactions between female and pH had significant effects on RMR (RMR in d^{-1}, ANCOVA, age: $F_{1, 277} = 196.82$, $p < 0.0001$, pH: $F_{2, 277} = 13.579$, $p < 0.0001$,

Table 1. Seawater carbonate chemistry parameters presented as the mean ± SD.

				Calculated			
Species	**rep**	**Nominal pH**	**MeasuredpH_T**	**Temp (°C)**	**pCO_2 (μatm)**	**Ωca**	**Ωar**
Amphiura filiformis							
	1	8.0	8.06 ± 0.02	11.20 ± 0.04	414 ± 17	3.39 ± 0.10	2.15 ± 0.06
		7.7	7.71 ± 0.06	11.14 ± 0.04	1044 ± 138	1.74 ± 0.24	1.10 ± 0.15
	2	8.0	8.04 ± 0.02	11.19 ± 0.03	435 ± 19	3.27 ± 0.12	2.07 ± 0.08
		7.7	7.64 ± 0.03	11.13 ± 0.04	1208 ± 91	1.43 ± 0.10	0.90 ± 0.06
	Mean	*8.0*	*8.05 ± 0.01*	*11.19 ± 0.02*	*425 ± 13*	*3.32 ± 0.08*	*2.11 ± 0.05*
		7.7	*7.67 ± 0.03*	*11.14 ± 0.03*	*1126 ± 83*	*1.58 ± 0.13*	*1.00 ± 0.08*
Strongylocentrotus purpuratus							
Female 1	1	8.0	8.05 ± 0.02	11.06 ± 0.28	418 ± 24	3.37 ± 0.15	2.14 ± 0.09
		7.7	7.72 ± 0.05	11.08 ± 0.20	1011 ± 108	1.76 ± 0.23	1.12 ± 0.14
		7.3	7.23 ± 0.06	10.95 ± 0.32	3309 ± 471	0.59 ± 0.08	0.37 ± 0.05
	2	8.0	8.02 ± 0.03	11.00 ± 0.23	464 ± 38	3.15 ± 0.19	2.00 ± .12
		7.7	7.71 ± 0.03	12.63 ± 0.09	1005 ± 82	1.76 ± 0.13	1.12 ± 0.08
		7.3	7.36 ± 0.04	10.68 ± 0.12	2339 ± 197	0.77 ± 0.08	0.48 ± 0.05
	Mean	*8.0*	*8.04 ± 0.02*	*11.03 ± 0.20*	*441 ± 22*	*3.26 ± 0.12*	*2.07 ± 0.08*
		7.7	*7.72 ± 0.04*	*11.59 ± 0.26*	*1009 ± 75*	*1.76 ± 0.15*	*1.12 ± 0.10*
		7.3	*7.29 ± 0.04*	*10.83 ± 0.18*	*2868 ± 301*	*0.67 ± 0.06*	*0.42 ± 0.04*
Female 2	1	8.0	8.06 ± 0.02	10.80 ± 0.28	415 ± 22	3.36 ± 0.15	2.12 ± 0.09
		7.7	7.72 ± 0.11	11.80 ± 0.61	1059 ± 248	1.92 ± 0.55	1.22 ± 0.35
		7.3	7.20 ± 0.04	12.07 ± 0.40	3466 ± 362	0.58 ± 0.05	0.36 ± 0.03
	2	8.0	7.98 ± 0.08	10.94 ± 0.25	589 ± 171	3.04 ± 0.32	1.93 ± 0.20
		7.7	7.67 ± 0.03	11.33 ± 0.41	1114 ± 70	1.53 ± 0.09	0.97 ± 0.06
		7.3	7.31 ± 0.07	11.10 ± 0.26	2751 ± 301	0.77 ± 0.18	0.49 ± 0.12
	Mean	*8.0*	*8.02 ± 0.04*	*10.87 ± 0.18*	*502 ± 86*	*3.20 ± 0.17*	*2.02 ± 0.11*
		7.7	*7.69 ± 0.05*	*11.52 ± 0.33*	*1092 ± 100*	*1.68 ± 0.22*	*1.07 ± 0.14*
		7.3	*7.26 ± 0.04*	*11.55 ± 0.25*	*3085 ± 245*	*0.68 ± 0.10*	*0.43 ± 0.06*
Overall		*8.0*	*8.03 ± 0.02*	*11.02 ± 0.09*	*458 ± 32*	*3.26 ± 0.08*	*2.06 ± 0.05*
		7.7	*7.69 ± 0.02*	*11.39 ± 0.13*	*1078 ± 48*	*1.67 ± 0.09*	*1.06 ± 0.06*
		7.3	*7.27 ± 0.03*	*11.25 ± 0.18*	*2993 ± 188*	*0.68 ± 0.04*	*0.43 ± 0.04*

Seawater pH on the total scale (pH_T), temperature (*T*; °C), salinity of 32, and total alkalinity of 2386 μmol kg^{-1} were used to calculate CO_2 partial pressure (pCO_2) and saturation of carbonate species (Ωca and Ωar).

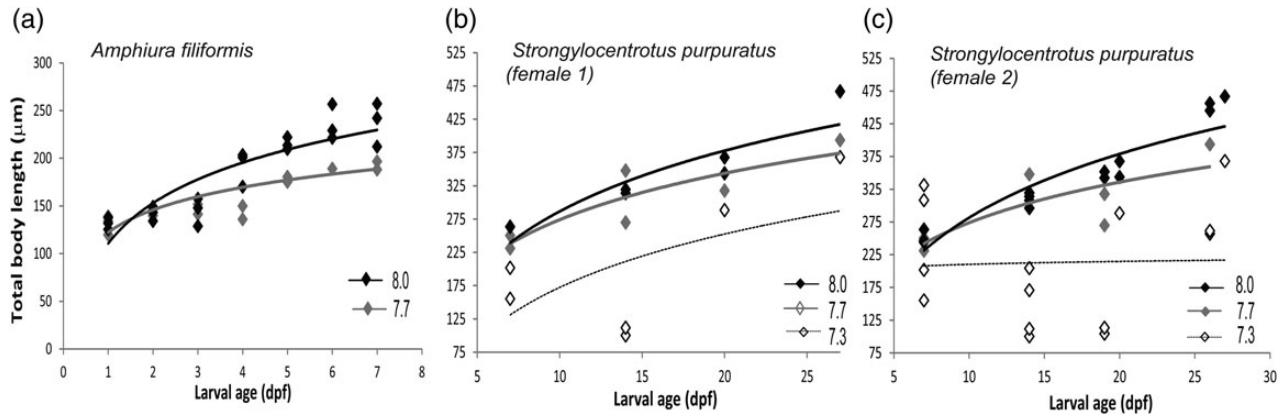

Figure 2. Increase in total body length (TL in μm) over time (day post-fertilization, dpf) and significant logarithmic relationships per pH treatments in (a) *A. filiformis* (1–7 dpf), (b and c) *S. purpuratus* (females 1 and 2, 7–27 dpf).

female: $F_{1,\ 277} = 16.786$, $p < 0.0001$, pH × female: $F_{2,\ 277} = 11.654$, $p < 0.0001$; RMR in μm^{-1}, ANCOVA, age: $F_{1,\ 277} = 138.3362$, $p < 0.0001$, pH: $F_{2,\ 277} = 45.07$, $p < 0.0001$, female: $F_{1,\ 277} = 16.683$, $p < 0.0001$, pH × female: $F_{2,\ 277} = 9.874$, $p < 0.0001$). When comparing within treatments, there was no significant difference in RMR between the two females in the control condition (*t*-test, $p = 0.458$). Larvae of female 1 reared at pH 7.7 had significantly higher RMR than those of female 2 (*t*-test, $F_{1,\ 83} = 12.509$, $p = 0.001$). Larvae of female 2 reared at pH 7.3 had a significantly higher RMR than those of female 1 (*t*-test, $F_{1,\ 92} = 4.107$, $p = 0.046$).

Effects of decreased pH on larval swimming

In larval *A. filiformis*, larval age had significant effects on gross swimming speed, and net vertical and horizontal velocities, with

Table 2. Statistical output from ANCOVA for swimming metrics of larval *A. filiformis* and *S. purpuratus* with age as a covariate.

Source	Parameter	d.f.	MS	*F*-value	*p*-value
Amphiura filiformis					
Age	Gross speed	1	88 472.82	31.80	<0.0001
	Horizontal velocity	1	158.63	0.09	0.76
	Vertical velocity	1	78 144.16	19.29	<0.0001
pH	Gross speed	1	2474.67	0.89	0.35
	Horizontal velocity	1	20 987.63	11.96	<0.0001
	Vertical velocity	1	5611.11	1.39	0.24
Error	Gross speed	153	2782.31		
	Horizontal velocity	153	1755.25		
	Vertical velocity	153	4051.27		
Strongylocentrotus purpuratus (Female 1)					
Age	Gross speed	1	137 227.27	37.69	0.00
	Horizontal velocity	1	454 551.54	181.88	0.00
	Vertical velocity	1	1141.75	0.21	0.64
pH	Gross speed	2	3613.94	0.99	0.37
	Horizontal velocity	2	6960.75	2.79	0.06
	Vertical velocity	2	1814.82	0.34	0.71
Error	Gross speed	293	3641.34		
	Horizontal velocity	293	2499.13		
	Vertical velocity	293	5367.72		
Strongylocentrotus purpuratus (Female 2)					
Age	Gross speed	1	77 559.97	20.59	0.00
	Horizontal velocity	1	159 490.34	57.80	0.00
	Vertical velocity	1	702.12	0.14	0.71
pH	Gross speed	2	13 806.01	3.66	0.03
	Horizontal velocity	2	4362.54	1.58	0.21
	Vertical velocity	2	19 023.00	3.78	0.02
Error	Gross speed	203	3767.35		
	Horizontal velocity	203	2759.43		
	Vertical velocity	203	5039.12		

Table 3. Statistical output from ANCOVA for swimming metrics of larval *A. filiformis* and *S. purpuratus* with calculated total body length (TBL) as a covariate.

Source	Parameter	d.f.	MS	*F*-value	*p*-value
Amphiura filiformis					
TBL	Gross speed	1	94 782.19	34.58	<0.0001
	Horizontal velocity	1	1468.88	0.84	0.36
	Vertical velocity	1	92 886.80	23.49	<0.0001
pH	Gross speed	1	50 077.19	18.27	<0.0001
	Horizontal velocity	1	6908.54	3.96	0.05
	Vertical velocity	1	58 387.91	14.76	<0.0001
Error	Gross speed	153	2741.07		
	Horizontal velocity	153	1746.68		
	Vertical velocity	153	3954.92		
Strongylocentrotus purpuratus (Female 1)					
TBL	Gross speed	1	128 918.22	35.13	0.00
	Horizontal velocity	1	402 301.46	150.26	0.00
	Vertical velocity	1	486.59	0.09	0.76
pH	Gross speed	2	50 948.92	13.88	0.00
	Horizontal velocity	2	96 019.15	35.86	0.00
	Vertical velocity	2	1579.61	0.29	0.75
Error	Gross speed	293	3669.70		
	Horizontal velocity	293	2677.46		
	Vertical velocity	293	5369.96		
Strongylocentrotus purpuratus (Female 2)					
TBL	Gross speed	1	79 813.78	21.25	0.00
	Horizontal velocity	1	163 234.04	59.55	0.00
	Vertical velocity	1	946.91	0.19	0.67
pH	Gross speed	2	51 350.28	13.67	0.00
	Horizontal velocity	2	39 836.87	14.53	0.00
	Vertical velocity	2	10 301.34	2.04	0.13
Error	Gross speed	203	3756.25		
	Horizontal velocity	203	2740.99		
	Vertical velocity	203	5037.91		

swimming speeds generally decreasing as larvae aged (Table 2, Figure 4). However, pH treatment did not have significant effects on gross swimming speed nor net vertical velocities (ANCOVA, $F_{1,\ 153} = 0.89$ and 1.39, $p = 0.35$ and 0.24, respectively). Horizontal velocity was significantly higher among larvae reared under pH 8.0 ($F_{1,\ 153} = 11.96$, $p < 0.0001$). When controlling for differences in the growth rate between larvae growth under different conditions by using total body length as a covariate, pH treatment had significant impacts on all three swimming metrics observed: at a given size, larvae reared under pH 8.0 had significantly higher swimming speeds (gross, horizontal, and vertical) than those under pH 7.7 (ANCOVA, $F_{1,\ 153} \geq 3.96$, $p \leq 0.05$, Table 3).

In larval *S. purpuratus*, age had significant effects on gross swimming speed and horizontal swimming speed ($F \geq 0.21, p \leq 0.0001$) but not vertical velocities ($F \leq 0.21$, $p > 0.6$, Table 2) in larvae from both maternal lineages (females 1 and 2). Swimming of larval urchins was more horizontally directed as they aged such that gross speed increased without a corresponding increase in vertical velocity (Table 2, Figure 4). Using age as a covariate, the pH level affected the swimming speeds of larvae from the two maternal lineages differently. In female 1, pH had no significant effect on any of the three swimming metrics ($F_{2,\ 203} \leq 3.79$, $p > 0.34$). In female 2, larvae reared under decreased pH conditions had higher gross swimming speeds and vertical velocity ($F_{2,\ 203} \geq 3.66$, $p < 0.03$). However, pH treatments had no significant effect on horizontal velocity ($p = 0.21$). These differences in how larval swimming speeds respond to pH treatments between the two maternal lineages was removed when total body length was used as a covariate (Table 3). In larval urchins from both females 1 and 2, total larval body length had significant effects on gross speed and horizontal velocity ($F \geq 25.25$, $p < 0.001$). The pH level significantly affected gross swimming speed and horizontal velocity ($F \geq 13.67, p < 0.0001$); however, vertical velocity was not affected ($F \leq 2.04, p > 0.13$).

Discussion

We compared larval sensitivity to reduced pH of two echinoderm species that naturally experience low pH as adults but different pH environments as larvae. Both echinoderm species experienced elevated mortality and reduced growth rates when exposed to decreased pH. However, the effects of decreased pH differed between these two species, and even between familial groups of *S. purpuratus*. The presence of both inter- and intraspecific variations in responses of two important echinoderm species to reduced pH has significant implications for adaptive potential and ecosystem responses to global climate change.

Interspecific variability: larval brittlestars are highly sensitivity to reduced pH

Larval *A. filiformis* experienced a greater mortality rate at decreased pH when compared with *S. purpuratus*, with an average of 20% survivorship after 6 d compared with 25% after 27 d for *S. purpuratus*. Such high larval mortality over short durations suggests that numbers of successful recruits may be significantly reduced under

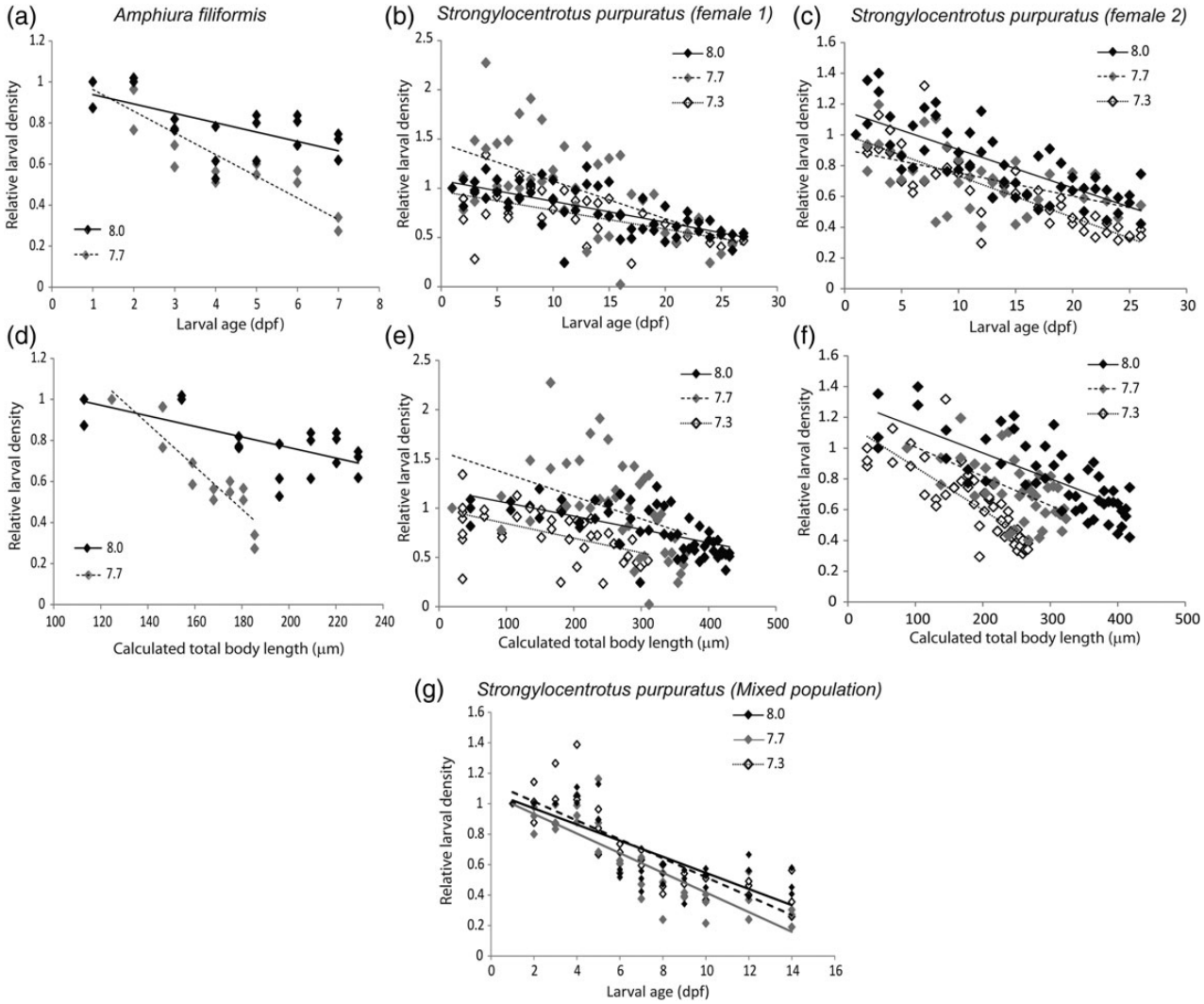

Figure 3. Changes in relative larval densities, calculated based on duplicate 10 ml subsamples, over time (day post-fertilization, dpf) for (a) larval *A. filiformis* and (b and c) *S. purpuratus* (females 1 and 2). The RMR was computed as the coefficient of significant linear regression. To correct for the difference in the growth rate, changes in relative larval densities were also plotted against total body length calculated using regression equations shown in Figure 2.

prolonged exposure. Decreased pH reduced the larval growth rate of both species in our study. This observation is consistent with previous studies on other echinoderm larvae (Stumpp *et al.*, 2011b; Dorey *et al.*, 2013; Dupont and Thorndyke, 2013). However, the magnitudes of pH effects on larval growth differed between species. A 100% decrease in the growth rate between pH 8.0 and 7.7 was observed for *A. filiformis*, whereas less than a 40% decrease between the three tested pH levels (8.0, 7.7, and 7.3) was observed for *S. purpuratus* (Figure 3, Supplementary Table S2). *Amphiura filiformis* is highly abundant in the muddy substratum of the North Sea, playing important roles in bioturbation and hence in biogeochemical cycling I. It is also an important food source for various flat fish and crabs (O'Connor *et al.*, 1983). If the growth rate and survivorship observed in the laboratory under low pH are indicative of natural *A. filiformis* population under future OA conditions, negative responses of this key ecosystem engineer could lead to significant impacts on energy transfer and other aspects of benthic community structure.

Given larval swimming is tightly coupled to the biomechanical limitations imposed by larval morphologies (Chan, 2012), we considered the differences in sizes between pH treatments when assessing their impacts on swimming (Figure 4, Table 3). When corrected for size, pH had significant effects on all swimming metrics, such that larval brittlestars in low pH swam significantly slower (gross speed and horizontal and vertical velocity). In contrast, reduced pH had significant positive effect on size-corrected swimming of larval *S. purpuratus*, such that individuals reared under decreased pH had higher gross and horizontal speeds. One possible interpretation is that the two species employ different behavioural responses to similar pH levels, i.e. reduced swimming for larval brittlestars but increased swimming for larval urchins. Alternatively, this interspecific difference could imply larval urchins have a wider range of behavioural plasticity when challenged by reduced pH to maintain swimming. This could be a consequence of adaptation to different pH envelopes, in which *A. filiformis* is naturally exposed to a narrower range of pH when compared with *S. purpuratus*.

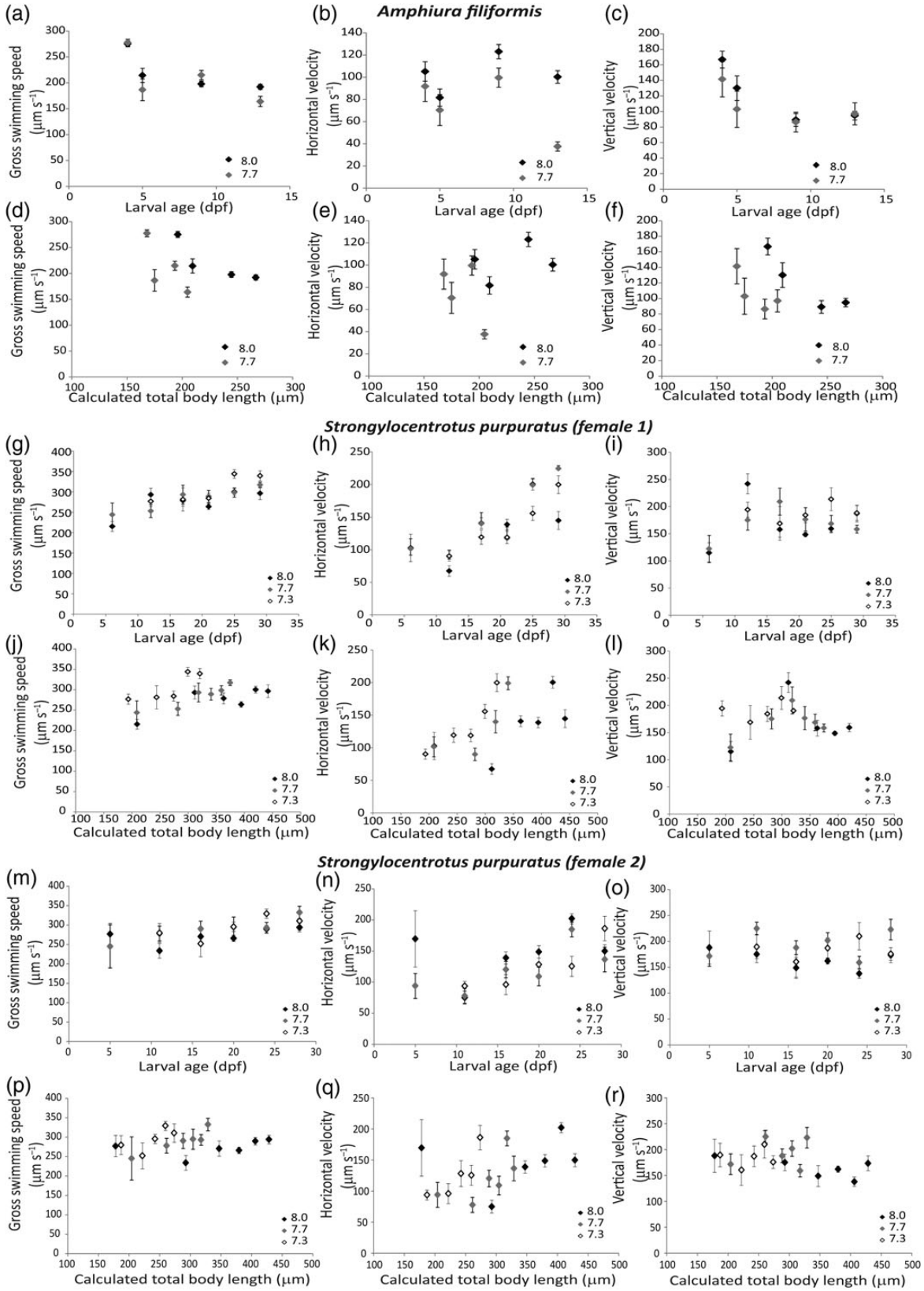

Figure 4. Swimming metrics, gross swimming speed and horizontal and vertical velocities (mean ± standard error) of larval *A. filiformis* (a–f) and *S. purpuratus* [female 1 (g–l) and female 2 (m–r)] plotted against larval age and calculated total body length.

Differences in sensitivity between the two tested species may reflect adaptation to different pH environment experienced during the larval stages (stable for *A. filiformis*, variable for *S. purpuratus*) rather than the pH environment experienced by adults (variable for both species). Beyond differences in larval exposure to low pH, further comparative studies are needed to better understand the roles of evolutionary history (e.g. ophiuroids vs. echinoids), maternal investment [e.g. egg size of *A. filiformis* is ~60 μm and *S. purpuratus* is ~80 μm (Bowner, 1982; Levitan, 1993)], and timing of life-history events [e.g. spawning in late summer of *A. filiformis* and spring for *S. purpruatus* (Bowner, 1982; Cochran and Engelmann, 1975)] in shaping an organism's ability to cope with pH stress. Such studies are essential for building a mechanistic understanding to predict community responses to OA.

Intraspecific variability: maternal lineage affects larval urchins' sensitivity

Effects of reduced pH on growth and swimming performance differed between the two maternal lineages of larval urchins studied. These differences could be explained by the difference in the mortality rate between larvae from females 1 and 2. Highly synchronized budding was observed in a large fraction of larval urchins of female 1 reared under pH 7.7, but not in female 2. Most released buds did not grow into functional individuals. The transitory increases of larval density due to the release of buds led to a higher estimate of the larval mortality rate, which is the slope of a significant linear regression of all the density count over time. Similar maternal differences in timing and frequencies of larval cloning have also been reported in larval sand dollars exposed to fish mucus (Vaughn, 2009, 2010).

Age-specific swimming metrics of larval *S. purpuratus* were affected by pH differently in the two maternal lineages: no significant effects on larvae from female 1, but significantly higher gross speeds and vertical velocities in larvae from female 2 (Table 3, Figure 4). These differences are likely due to the different growth pattern caused by the size reduction during budding, because these differences disappeared when developmental delays were taken into account. When corrected for size, larval urchins from both lineages had significantly higher gross speeds and horizontal velocities but not vertical velocities when reared under decreased pH. This effect of pH on size-corrected larval swimming also highlights the multifaceted potential impacts of pH, both directly through changes in behavioural choices and indirectly through altered morphologies that in turn alter swimming biomechanics.

Larval urchins demonstrate plasticity during acute exposure

Our observations were limited to short-term exposure to reduction in pH in the laboratory. Hence, generalization of our results to how larval urchins might response to chronic exposure to OA conditions under future field conditions should be approached cautiously. The observed plasticity in larval urchin response in growth and swimming appeared to be strategies to cope with stressful conditions during short-term exposure, but are not necessarily beneficial to surviving prolonged exposure. Nonetheless, our observations suggest hypotheses about the natural selection of responses to low pH by an important urchin species.

The budding we observed did not lead to numerical increases in viable larval population under laboratory conditions, because the buds released did not survive and develop into functional larvae. If budding has a selective value as an OA response, it may lead to numerical increases under different acidification conditions. An alternative hypothesis is that budding may act as a mechanism for size reduction. Size reduction could be beneficial to larvae in the short term, e.g. by reducing respiratory demands, but are likely to have legacy effects on later larval and juvenile stages that are at least partly deleterious (Chan *et al.*, 2013). Observed changes in larval urchin gross swimming speed did not translate into changes in vertical velocity. Maintenance of vertical velocity under reduced pH conditions could help larvae retain capacity to regulate their vertical positions in the water column. Larvae are known to use vertical swimming to regulate exposure to food, predators, stresses, such as UV and other environmental variations (Pennington and Emlet, 1986), and to influence lateral transport due to advection in ambient currents (Metaxas and Saunders, 2009; Miller and Morgan, 2013). Because some depth strata with unfavourable pH may nonetheless have favourable ambient currents and turbulence levels, larvae may confront trade-offs between reducing transport losses in a short term. However, chronic low pH exposure could slow development; assuming larvae are transported by the same current, they may reach their settlement sites prematurely at a small size or miss suitable habitat due to the increase in dispersal distance with longer pelagic larval duration.

In summary, exposure to reduced pH had overall negative impacts on larval echinoderms but sensitivity varied greatly between species. Interspecific variations suggest that ecological interactions between species and community structure could be altered due to the differential responses. For some species, large variation such as that observed between lineages of *S. purpuratus* could be a basis for future selective evolution, conferring resilience under future climate conditions.

Supplementary data

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

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