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RESEARCH ARTICLE

Effects of ocean-acidification-induced morphological changes on larval swimming and feeding

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SUMMARY

Reduction in global ocean pH due to the uptake of increased atmospheric CO_2 is expected to negatively affect calcifying organisms, including the planktonic larval stages of many marine invertebrates. Planktonic larvae play crucial roles in the benthic–pelagic life cycle of marine organisms by connecting and sustaining existing populations and colonizing new habitats. Calcified larvae are typically denser than seawater and rely on swimming to navigate vertically structured water columns. Larval sand dollars *Dendraster excentricus* have calcified skeletal rods supporting their bodies, and propel themselves with ciliated bands looped around projections called arms. Ciliated bands are also used in food capture, and filtration rate is correlated with band length. As a result, swimming and feeding performance are highly sensitive to morphological changes. When reared at an elevated P_{CO_2} level (1000 ppm), larval sand dollars developed significantly narrower bodies at four- and six-arm stages. Morphological changes also varied between four observed maternal lineages, suggesting within-population variation in sensitivity to changes in P_{CO_2} level. Despite these morphological changes, P_{CO_2} concentration alone had no significant effect on swimming speeds. However, acidified larvae had significantly smaller larval stomachs and bodies, suggesting reduced feeding performance. Adjustments to larval morphologies in response to ocean acidification may prioritize swimming over feeding, implying that negative consequences of ocean acidification are carried over to later developmental stages.

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Key words: ocean acidification, invertebrate larvae, swimming performance, sublethal effect, functional morphology, biomechanics.

INTRODUCTION

Atmospheric partial pressure of carbon dioxide (P_{CO_2}) has increased by over 36% since the 18th century, and over one-third of the anthropogenic CO_2 released to the atmosphere has been absorbed by the surface oceans (Sabine et al., 2004). The P_{CO_2} level in surface waters has increased by 100 parts per million (ppm) over the past century, and is predicted to continue rising at a faster rate than has been observed in the last 300 million years (Caldeira and Wickett, 2003). Ocean acidification (OA) is the reduction of ocean pH as this CO_2 is hydrated into seawater. Modeling studies suggest that pH levels in the surface ocean will drop by 0.3–0.4 pH units by 2100 (Orr et al., 2005).

A growing body of research suggests this pH drop will affect the physiologies of marine organisms (Pörtner, 2008) and, subsequently, their ecological functions and interactions with other organisms (Widdicombe and Spicer, 2008). In particular, OA is expected to negatively impact calcifying organisms by reducing carbonate saturation states (Fabry et al., 2008). Previous studies have investigated the impacts of OA on various calcifying organisms such as reef-building corals (Langdon et al., 2000), coralline algae (Hall-Spencer et al., 2008), cocolithophores (Riebesell et al., 2000), pteropods (Fabry et al., 2008), molluscs (Gazeau et al., 2007) and echinoids (Wood et al., 2008). These studies have demonstrated that OA could have negative impacts on growth, reproduction and development in a broad spectrum of calcifying marine organisms (Doney et al., 2009).

Planktonic larvae of many marine invertebrates also possess calcified structures, including shells of molluscan veligers, skeletal rods of echinoplutei and posterior ossicles of auricularia. These calcifying larval stages are likely to be sensitive to changes in ocean pH, and some species are known to be highly sensitive (Dupont and Thorndyke, 2009; Kurihara, 2008). For example, a 0.4 pH decrease caused 100% mortality in a larval brittle star, *Ophiothris fragilis* (Dupont et al., 2008). Experimental observations of larvae of other species suggest that reduced pH can have sublethal effects, including decreased larval growth and calcification rates and changes in gene expression patterns (Clark et al., 2009; Kurihara and Shirayama, 2004; O'Donnell et al., 2010). Future environmental stresses, including shifts in ocean pH, are likely to be expressed in many species initially as sublethal effects rather than outright mortality. Predicting ecological responses for marine communities to environmental changes therefore requires understanding the functional significance of these sublethal responses of larval stages.

Planktonic larvae of sessile marine invertebrates are the primary dispersal vectors that connect and sustain disjunct populations, and permit colonization of new habitats (Cowen et al., 2000; Gaines and Roughgarden, 1985; Underwood and Fairweather, 1989). Most planktonic larvae are not passive drifters; instead, they use active swimming to regulate their vertical positions in the water column (Chia et al., 1984; Metaxas, 2001; Young, 1995). Because environmental variables such as temperature, direction and strength of advective currents, and concentrations of prey and predators are

vertically structured in most marine environments, larval swimming behaviors can significantly affect larval survival and dispersal (North et al., 2008).

Larval swimming behaviors are constrained by morphological characteristics, such as size, shape and propulsive mechanism (Emlet, 1990; Emlet, 1991). For 'armed larvae' – larvae such as echinoplutei that use slender ciliated body extensions for swimming and/or feeding – the length and arrangement of ciliated extensions significantly affects both the weight distribution and the generation of propulsive force, and hence important aspects of swimming performance such as upward swimming speed and weight-carrying capacity (Grünbaum and Strathmann, 2003).

One key metric of larval swimming performance is stability, i.e. the ability to maintain swimming orientation in spite of perturbations from ambient flows. Experimental removal of the calcite skeleton of echinoplutei suggested that larval weight distribution depends strongly on the presence of calcite and that skeletons enhance larval stability by acting as counter-weights (Pennington and Strathmann, 1990). Hydrodynamic models have been used to argue that adjustments in arm number, length and elevation angle also strongly affect larval stability (Grünbaum and Strathmann, 2003; Clay and Grünbaum, 2010; Clay and Grünbaum, 2011). Arm elevation angle is the angle between an arm rod and its projection on the horizontal plane. Hence, a shorter distance between tips of paired arms implies a higher arm elevation angle. Their model predicted that higher arm elevation angles improve stability, minimizing movement towards downwelling water when larvae are exposed to vertical shear (Grünbaum and Strathmann, 2003). Motion analysis of echinoderm larvae from different taxa conformed to these predictions about larval stability (Strathmann and Grunbaum, 2006). Although the impacts of morphological changes on swimming performance are sublethal, they might nonetheless strongly impact adult population dynamics by altering larval dispersal and survival.

In addition to swimming, morphological changes amongst larvae have implications for other ecological functions. In echinoplutei, ciliated bands looped around larval arms are used both for generating propulsive forces and for filtering food particles. Maximum clearance rate in these larvae increases with the length of the ciliated bands (Hart, 1991). Echinoplutei are known to be phenotypically plastic: larvae develop longer arms when food is limited than when food is abundant (Hart and Strathmann, 1994; Miner, 2005). However, there is a morphological trade-off involving arm length: although longer larval arms increase ciliated band length and enhance feeding efficiency, longer larval arms can also incur the cost of reduced stability in moving water. This is because longer arms cut across more flow lines and increase the destabilizing torque larvae experience, potentially exacerbating tendencies to move into downwelling water or to tumble (Strathmann and Grünbaum, 2006).

In addition to its effect on arm length, food concentration also affects stomach size among echinoids. Under food-limited conditions, larvae develop smaller stomachs and rudiments. Rudiments are united structures from the amniotic invagination and hydrocoel that give rise to future adults (Hart and Strathmann, 1994). Plasticity in larval stomach size is unlikely caused by food distending the stomach because larval urchins develop bigger stomachs when reared under high food concentrations even before they are capable of particle capture (Miner, 2005). This observation suggests that cues of food availabilities induce changes in morphogenesis of both larval arms and stomachs. Heyland and Hodin further hypothesized that the thyroid hormone from algal food serves as a signaling cue to accelerate development and initiate metamorphosis (Heyland and Hodin, 2004). Collectively, these studies strongly suggest that larval nutrition and morphogenesis are tightly coupled.

The functional relationships between arm morphology, swimming performance and feeding success suggest the hypothesis that sublethal changes in larval morphologies observed in OA experiments (such as shorter larval arms) can reduce swimming performance, feeding success or both. Both swimming and feeding have implications for larval survival; degradation of performance in either function may be a mechanism through which effects of OA on larval morphology that are sublethal in the laboratory may nonetheless have significant consequences for wild populations.

In this study, we assessed the functional impacts of OA on swimming and feeding performance of larval sand dollars *Dendraster excentricus*, as indicated by larval swimming speeds, helical swimming geometries and stomach sizes. This sand dollar is a good model organism because swimming mechanics and behavior of this species have been previously described (Chan and Grünbaum, 2010; Clay and Grünbaum, 2010; Clay and Grünbaum, 2011). Larval stomach size of sand dollars has also been shown to positively correlate with clearance rates, and therefore can be used as a proxy for feeding performance (Hart and Strathmann, 1994). Because within-population variation in sensitivity to changes in pH has been suggested for other echinoids (Byrne et al., 2010), we compared the sublethal responses of larvae from four maternal lineages. For each of these lineages, we reared the larvae under two P_{CO_2} concentrations, documented the corresponding larval morphologies and quantified swimming performance with non-invasive video tracking techniques.

MATERIALS AND METHODS

CO_2 incubations

Larval *Dendraster excentricus* Eschscholtz 1831 were raised under two different concentrations of P_{CO_2}, chosen to reflect CO_2 emissions scenarios predicted by the Intergovermental Panel on Climate Change: 380 ppm (present-day atmospheric CO_2 level) and 1000 ppm (A1FI 'business as usual scenario') (IPCC, 2007). Seawater was 0.22 μm-filtered and batch-equilibrated with gas blends, prepared by combining pure CO_2 and CO_2-free air to reach 380 and 1000 ppm. Hereafter, we refer to these two treatments as 'present-day' and 'acidified', respectively. We measured the pH of each culture vessel twice a day using an Orion 720A meter and a ROSS Ultra combination electrode calibrated with Nation Bureau of Standards (NBS) buffers (Thermo Scientific Corp., Beverly, MA, USA). Water samples from the batch equilibration buckets were collected and fixed in accordance with SOP1 of Dickson et al. (Dickson et al., 2007) at the beginning and middle of the experiment. Samples were analyzed for total alkalinity (A_T) and total dissolved inorganic carbon (DIC) by NOAA's Pacific Marine Environmental Laboratory. Analysis of these samples showed that the experimental conditions matched the expected values closely, with mean pH values of 8.06 and 7.75, respectively. The present-day treatment at the beginning and middle of the experiment had A_T values of 2124.68 and 2113.63 μmol kg^{-1} and DIC values of 1892.3 and 1889.5 μmol kg^{-1}, respectively. The acidified treatment at the beginning and middle of the experiment had A_T values of 2126.95 and 2108.57 μmol kg^{-1} and DIC values of 2030.8 and 2023.6 μmol kg^{-1}, respectively.

Larval cultures

Adult *D. excentricus* were collected from Crescent Beach, East Sound, Orcas Island, WA, USA, in early summer 2009 and

maintained in sea tables at Friday Harbor Laboratories until use. Spawning was induced by injecting 0.5–1 ml of 0.55 mol l^{-1} KCl into the coelomic cavity (Strathmann, 1987). Collected eggs were washed through a 200 µm sieve placed in a beaker with 1000 ml of 0.22 µm-filtered seawater and fertilized with five drops of diluted sperm. Ten minutes after fertilization, the eggs were examined under a microscope for the presence of fertilization envelopes to confirm fertilization success. Four maternal lineages were created by fertilizing eggs from four females with sperm from a single male, and were labeled as females 1 to 4. Egg sizes did not differ significantly between females (mean ± s.e.m. diameter=123.8±8.1 µm, N=40; ANOVA, $F_{3,36}$=1.73, P=0.177).

Ten hours post-fertilization, hatched gastrulae were transferred into 24 glass jars, each 3.7 l, providing three replicates for each maternal lineage within each of the two CO_2 treatments. Because movement of mesenchyme cells occurs post-gastrulation (Decker and Lennarz, 1988), exposure at hatching enabled us to assess the effects of CO_2 treatment on larval development and swimming throughout spicule and endoskeleton formation. Our experimental protocol implies that our study may underestimate the overall functional impact of OA, because other developmental processes such as fertilization and cleavage took place at the present-day P_{CO_2} level.

Larvae were reared under gentle bubbling with the respective pre-mixed CO_2 gases at 20±1°C in 0.22 µm-filtered seawater at a density of approximately 2 individuals ml^{-1}. Water was changed every other day with present-day and acidified seawater. Larvae were fed with an equal mixture of the algae *Rhodomonas lens* and *Dunaliella tertiolecta* at 5000 cells ml^{-1}. After algal concentration was determined with a haemocytometer, cells were concentrated by centrifugation and subsequently resuspended in filtered seawater from the respective CO_2 treatments. Food was added 12 h prior to water changes to minimize the effect of photosynthesis by algal food on carbonate chemistry.

Behavioral observations and video processing

To assess the functional impact of exposure to elevated P_{CO_2} through larval development, we observed swimming behaviors of four-, six- and eight-arm larvae from the two pH treatments and four maternal lineages using video tracking analysis, following the methods of Chan and Grünbaum (Chan and Grünbaum, 2010). In brief, approximately 300 larvae from each treatment (~100 larvae from each replicated culturing jar) were pipetted into one of four Plexiglas® chambers (3.5×3.5×30 cm) submerged in a common water bath at a constant temperature (20°C). Larvae from different pH treatments were observed in filtered seawater equilibrated to their respective rearing P_{CO_2} levels. Larvae were allowed to acclimate for 10 min, and then were gently stirred. After an additional 5 min for transient water movement to dissipate prior to filming, movements of individuals were recorded under infrared illumination with digital camcorders (Panasonic DS400, Secaucus, NJ, USA) at 15 frames s^{-1}. Video clips were captured in a series of vertical 'casts' that sequentially imaged lower (0–14.4 cm) and upper (11.6–25 cm) regions of each chamber. Each cast took 10 min: 3 min videos captured in each region of each chamber, 3 min to move cameras, plus 1 min pause. Six casts were performed in each observation. Observations were replicated three times for each developmental stage (four-, six- and eight-arm stages). Under this sampling scheme, larvae from each maternal lineage in each pH treatment had a total of 18 casts at each developmental stage.

We also tested for swimming responses to short-term changes in P_{CO_2} in four-arm larvae from two of the maternal lineages reared under present-day conditions. We exposed these larvae to either present-day or acidified seawater for 60 min, while observing their swimming behaviors using the video protocol described above.

Video clips were analyzed by equalizing lighting, removing background variations, distinguishing moving larvae based on brightness and size, and extracting the pixel coordinates of larvae using a customized version of the open-source Linex-based video editing package avidemux2.4. Videos were calibrated using a grid of 2×2 mm squares, with 2 mm between each square. Pixel coordinates were converted to physical positions and assembled into larval swimming trajectories using Tracker3D, an in-house MATLAB program (Fig. 1) (for details, see Clay and Grunbaum, 2010).

Morphometric analysis

At the end of each observation, larvae were relaxed in $MgCl_2$ and fixed in buffered 2% formaldehyde. Individuals were photographed under a microscope at 16× using a Nikon E4500 digital camera. Arm lengths, distances between pairs of arms, body lengths and widths, and stomach lengths and widths of 10 individuals from each observation chamber were measured with ImageJ (Abramoff et al., 2004) (two pH treatments × four maternal lineages × three replicated observations × three stages × 10 individuals, total N=720), using photographs of a stage micrometer for calibration (Fig. 2). Projected stomach size was computed by assuming the stomach is an ellipsoid (Heyland and Hodin, 2004).

Because the observed morphological characteristics are correlated, we performed a principal component analysis (PCA) on the correlation matrix of morphological characteristics at each stage with varimax rotation using Kaiser normalization to identify key components (Harris, 1975). The number of factors to retain was determined by eigenvalues and the shape of the scree plot. Because larval *D. excentricus* add arms as they develop, the number of observed morphological characteristics increased with time. Though larvae were reared in separate jars, they were pooled prior to their video observation and fixation. Therefore, we could not assess jar effects, which are instead subsumed into the error term. We assessed the effect of the main factors, pH and maternal lineage, on larval morphology with a separate two-way ANOVA for each stage (Zar, 1996).

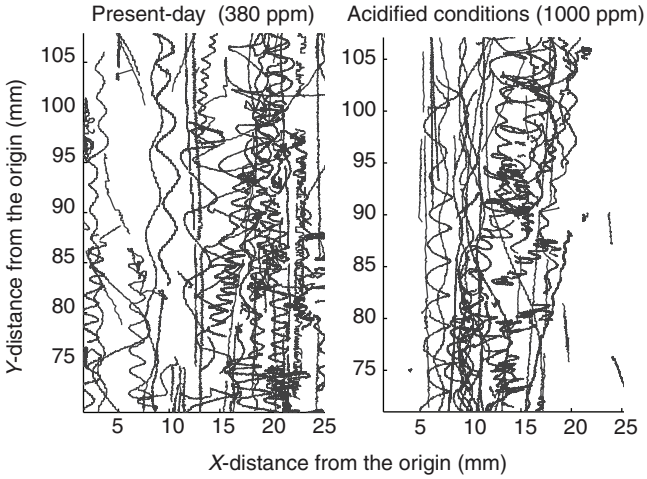

Fig. 1. Representative larval swimming trajectories under acidified and present-day conditions from 3 min video clips of eight-arm larval *Dendraster excentricus*. Size of the field of view is 25×35 mm. The bottom left-hand corner of each chamber is considered the origin.

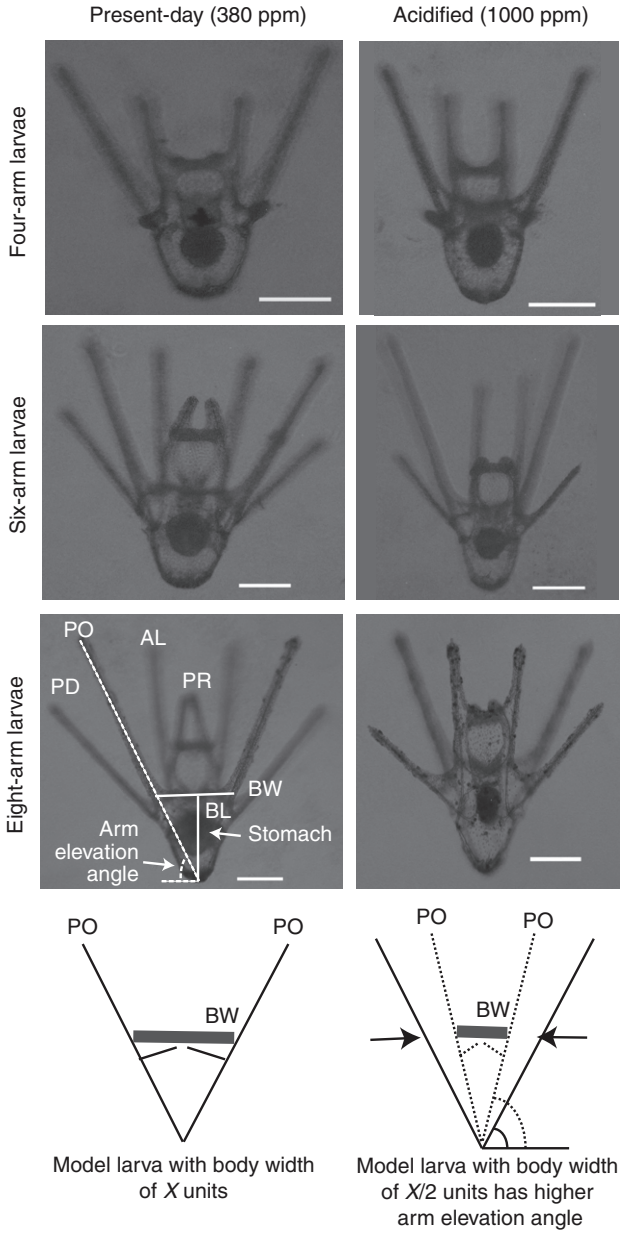

Fig. 2. Representative *D. excentricus* individuals from acidified and present-day treatments at four-, six- and eight-arm stages (top three rows). Measured morphological characteristics include body width (BW), body length (BL), stomach width, stomach length, lengths of anterolateral arms (AL), postoral arms (PO), preoral arms (PR) and posterodorsal arms (PD) and the distances between arm tips. Note that four-arm larvae have not developed PR and PD and six-arm larvae had not developed PR fully. Scale bars, 100 μm. The bottom row illustrates the geometric relationship between larval body width, arm length and arm elevation angle.

Swimming performance analysis

Our analysis of swimming metrics followed the methodology of Chan and Grübaum (Chan and Grübaum, 2010). To avoid including passive particles in our analysis, only larval trajectories longer than 15 s and having horizontal acceleration >0.06 mm s^{-2} in magnitude were included in the statistics. Individual trajectories were further subdivided into net up-swimmers and down-swimmers. We applied cubic smoothing splines with different knot spacings to remove frame rate noise while defining each larval swimming trajectory (hereafter, path) and to identify the overall direction of travel (hereafter, vertical axis of travel).

Larval sand dollars typically swim in a helical manner. For each path, we computed the larva's total swimming speed by taking the first time derivative of the path, and the net vertical and horizontal velocities by dividing the displacement between the start and end points of the axis of travel by the path duration. Two-dimensional (2-D) projections of helical paths, which appear as sinusoids, underestimate horizontal movement. To compensate for this underestimate, we applied a correction factor of $\pi/2$ to mean horizontal speed components. The vertical axes of the helices represent the overall direction of travel and the actual larval paths oscillated along these axes. To quantify the oscillating nature of path, we also computed oscillatory speed as the time derivative of the distance between the path and the vertical axis. We used the maximum horizontal distance between the path and the axis as a measure of helical width. We further characterized the shapes of swimming trajectories by quantifying zero crossings, defined as points at which the path and the axis intersect. The distance between zero crossings is a measure of helical pitch [see fig. 2 in Chan and Grünbaum (Chan and Grünbaum, 2010)].

We computed weighted means of swimming metrics for each vertical cast by sampling at 5 s intervals and taking the mean of swimming metrics of all larval trajectories observed at those time points. This scheme gives equal statistical weight to each frame in which each larva was tracked, avoiding bias towards shorter or longer trajectories. Initial analysis showed that the mean swimming speeds of larval sand dollars in both treatments were approximately 0.3 mm s^{-1}, similar to the value reported by Podolsky and Emlet (Podolsky and Emlet, 1993). Assuming an even distribution at the beginning of the experiment, we estimated that most larvae would have left the field of view (0–25 cm) within the first 15–20 min of observation. To avoid a bias towards slow-swimming individuals, we subsequently used only data collected from the first two casts (i.e. the first 20 min of the experiment) in the statistical comparisons.

The effects of CO_2 treatment, maternal lineages and their interactions on larval swimming performance were tested with a two-way analysis of covariance (ANCOVA) with developmental stage as a covariate. In the experiment on four-arm larval responses to short-term changes in P_{CO_2} level, a two-way ANOVA was used to test for effects of CO_2 treatment and maternal lineages. We performed *post hoc* Tukey's tests when significant differences ($P<0.05$) in component scores were detected (Zar, 1996). All statistical analyses were conducted in PASW 18.0 (IBM Corp., Armouk, NY, USA).

RESULTS

Morphometric analysis

Across all observed developmental stages, acidified larvae had narrower larval bodies than those reared in present-day treatments. In the six- and eight-arm stages, acidified larvae also had smaller larval stomachs. Maternal lineage and the interaction of CO_2 treatment with maternal lineage also had significant effects on morphologies in six- and eight-arm larvae (Tables 1, 2, Fig. 2). In particular, larval arms were shorter in the acidified treatments for six- and eight-arm offspring of females 1 and 2, but not offspring of females 3 and 4 (Table 1).

In four-arm larvae, PCA axis 1 was most correlated to the width of the larval body and distance between tips of pairs of arms (Table 2). PCA axis 2 was most correlated to stomach size. PCA axis 3 was most correlated to lengths of arms. Two-way ANOVA of the PCA component scores showed that acidified larvae had

Table 1. Morphometrics of four-, six- and eight-arm larval *Dendraster excenticus* (means ± s.e.m.; units are μm, except for stomach volume, which is ×1000 μm^3) from four maternal lineages and two CO_2 treatments (present-day at 380 ppm and acidified at 1000 ppm)

	Female 1		Female 2		Female 3		Female 4	
	380 ppm	1000 ppm	380 ppm	1000 ppm	380 ppm	1000 ppm	380 ppm	1000 ppm
Four-arm stage								
Width	172.4±3.5	157.9±4.1	167.0±4.2	158.0±3.6	164.1±2.6	150.3±3.6	162.2±3.8	176.5±3.1
Height	185.6±2.6	188.5±3.1	187.7±2.1	196.2±6.9	182.5±2.7	190.3±2.5	190.8±3.3	186.1±2.6
AL distance	124.7±9.5	96.3±6.5	101.3±7.4	91.9±6.5	108.3±6.6	78.3±7.8	86.5±5.4	102.6±4.8
PO distance	360.1±10.2	314.7±13.5	356.4±13.9	330.9±16.5	326.2±7.5	299.8±10.0	320.4±8.5	353.1±8.8
AL length	385.5±8.5	397.4±9.2	403.2±8.7	400.4±5.4	392.2±7.2	403.4±6.3	390.5±9.0	397.3±7.8
PO length	482.3±9.4	485.2±9.6	486.7±6.0	486.3±9.7	458.4±10.4	484.5±7.2	473.7±8.7	460.9±8.7
Stomach length	82.0±1.5	79.8±2.2	81.3±4.1	83.6±2.2	84.8±1.8	79.2±3.9	83.9±2.0	80.8±1.5
Stomach height	62.5±1.2	55.5±1.4	56.6±2.6	56.6±1.5	60.6±1.0	52.8±1.1	58.4±1.3	62.5±2.1
Stomach volume	16.2±0.50	14.02±0.62	14.85±1.42	15.02±0.42	16.24±0.54	13.21±0.73	15.53±0.62	15.96±0.22
Six-arm stage								
Width	168.9±3.3	152.3±3.3	182.9±4.5	146.4±4.4	178.6±2.2	161.6±2.6	181.0±2.5	172.9±3.8
Height	197.7±3.1	202.6±3.1	200.5±4.3	204.7±3.0	201.3±2.5	212.8±3.4	202.8±2.7	197.9±2.7
AL distance	110.1±5.0	94.9±6.1	134.1±7.7	92.5±6.5	122.2±5.5	113.9±5.9	125.4±3.7	132.5±5.3
PO distance	387.5±10.1	332.7±8.8	420.9±11.2	342.8±12.9	403.5±6.7	355.4±10.4	428.0±8.8	411.5±11.7
PD distance	340.8±13.1	321.5±14.1	389.5±16.0	303.6±11.2	336.8±8.4	388.0±11.2	415.8±12.4	426.9±10.9
AL length	433.7±9.2	460.1±8.2	470.9±11.7	459.4±11.1	466.7±7.0	498.6±8.6	475.1±9.5	477.9±7.6
PO length	554.8±10.4	531.4±15.1	566.3±13.5	592.7±9.0	558.5±6.4	580.7±12.2	591.8±9.1	565.6±10.1
PD length	211.4±7.1	203.8±8.8	248.6±13.9	214.6±8.9	224.3±8.1	240.1±6.5	258.3±6.3	265.7±5.4
Stomach length	84.6±2.7	72.4±1.9	88.4±2.7	77.2±1.8	80.2±1.6	87.0±1.9	89.8±2.2	93.6±3.6
Stomach height	65.2±2.5	51.7±1.3	77.3±5.7	58.8±2.1	65.2±2.7	62.9±2.0	69.0±2.2	69.6±2.9
Stomach volume	17.64±1.08	11.87±0.53	22.33±2.15	14.31±0.62	16.41±0.66	17.37±0.77	19.76±0.99	20.93±1.35
Eight-arm stage								
Width	193.9±3.5	182.7±3.9	208.8±5.5	181.3±3.9	196.5±3.4	174.4±2.9	197.5±3.5	205.3±4.0
Height	228.3±6.8	210.0±2.5	214.0±2.7	203.1±2.5	208.7±3.0	213.7±2.4	215.5±2.7	199.2±3.3
AL distance	172.1±3.7	151.8±5.9	187.2±6.6	150.7±4.8	161.2±4.9	157.2±5.2	163.1±4.0	188.5±5.0
PO distance	453.4±17.7	401.0±12.0	480.7±13.1	431.5±13.8	446.5±7.9	410.1±8.2	471.6±12.1	487.9±20.6
PR distance	42.8±11.9	38.7±1.9	36.0±2.7	29.2±1.9	29.8±2.5	28.3±1.9	33.5±3.1	45.2±11.0
PD distance	561.9±20.6	473.0±18.8	566.3±18.0	514.1±15.4	509.7±9.2	544.6±14.5	572.4±12.5	621.3±14.7
AL length	529.4±6.9	478.1±10.5	527.9±10.5	505.9±12.4	502.1±9.4	519.8±6.7	523.9±7.9	538.6±10.1
PO length	598.0±9.7	543.8±11.9	595.6±10.2	567.1±12.2	584.0±8.7	585.2±6.3	599.8±6.5	571.0±9.9
PR length	239.4±9.9	161.0±11.2	268.4±15.4	208.5±11.4	218.6±7.6	207.7±7.5	261.5±10.1	302.5±8.5
PD length	351.3±7.3	283.3±12.3	359.7±10.3	314.0±10.9	335.7±6.9	332.7±7.5	358.8±6.3	395.0±9.0
Stomach length	100.9±2.0	80.5±2.3	111.3±3.5	89.0±2.8	97.3±1.7	88.9±1.7	115.8±2.6	100.9±2.3
Stomach height	69.6±2.4	57.1±1.8	82.7±2.5	64.4±2.6	67.6±2.9	58.2±1.8	84.2±2.7	70.2±2.1
Stomach volume	22.38±1.13	14.66±0.78	29.48±1.64	18.38±1.12	20.92±1.14	16.40±0.7	31.07±1.59	22.49±1.07

AL, anterolateral arm; PD, posterodorsal arm; PO, postoral arm; PR, preoral arm.

narrower body widths (PCA axis 1 score, P=0.03), but there were no significant differences between the two CO_2 treatments in stomach size or arm lengths (PCA axes 2 and 3 scores). Maternal lineage alone did not have a significant effect on any of the PCA axis scores. However, there was a significant interactive effect of CO_2 treatment and maternal lineage on larval body width (PCA axis 1 score, P<0.001).

In six-arm larvae, PCA axis 1 was most correlated with larval body width, body length:width ratio and distances between tips of each pair of anterolateral and postoral arms. PCA axis 2 was most correlated to stomach size. PCA axis 3 was most correlated to the lengths of anterolateral and posterodorsal arms (Table 2). Two-way ANOVA of PCA component scores showed that acidified larvae had narrower larval bodies (PCA axis 1 scores, P<0.001) and smaller larval stomachs (PCA axis 2 scores, P=0.006), and had significantly shorter anterolateral and posterodorsal arm lengths (PCA axis 3 scores, P=0.009). Maternal lineage had significant effects on all three PCA component scores (Tables 1, 2): (1) larval body widths were wider in offspring of female 4 than in offspring of the other three females (PCA axis 1 scores, P<0.001); (2) stomach sizes of offspring of females 1 and 3 were smaller than those of the offspring of female 4 (PCA axis 2 scores, P=0.002); and (3) arm lengths of offspring of female 1 were shorter than those of offspring of female 4 (PCA axis 3 scores, P<0.001). There were also significant interactive effects of CO_2 treatment and maternal lineage on all three PCA axis scores (Table 2).

In eight-arm larvae, PCA axis 1 was most correlated with larval body width; distances between anterolateral, postoral and posterodosal arms; and lengths of anterolateral, posterodorsal and preoral arms. PCA axis 2 was most correlated to stomach size. PCA axis 3 was most correlated to length of larval body and body length:width ratio (Table 2). Two-way ANOVA of PCA component scores showed that acidified larvae had significantly smaller larval stomachs (PCA axis 2 score, P<0.001). However, CO_2 treatments did not significantly affect PCA axes 1 and 3 scores. To further investigate the effect of CO_2 treatments, we performed a two-way ANOVA on the raw measurements of individual morphological characteristics, after confirming that the data were normally distributed and had equal variance. Comparing morphological characteristics individually suggested that acidified larvae had significantly narrower bodies ($F_{1,233}$=23.569, P<0.001), shorter distances between tips of anterolateral and postoral arms ($F_{1,233}$≥6.33, P<0.01), and shorter preoral, postoral and posterodorsal arms ($F_{1,233}$≥11.8, P<0.001). Non-significance of differences between the two CO_2 treatments in PCA axis 1 scores was most likely driven by the lack of significant differences across treatments

Table 2. Effects of ocean acidification on larval morphologies for four-, six- and eight-arm larval *Dendraster excentricus*

	PCA 1	PCA 2	PCA 3
Four-arm larvae			
% of variance	38.23	19.13	13.85
Cumulative % of variance	38.23	57.36	71.21
Eigenvalue	3.82	1.913	1.385
Highest loading factors (component score)	Distance between AL (0.823) Body width (0.860) Distance between PO (0.734)	Stomach volume (0.975) Stomach width (0.822) Stomach length (0.768)	PO length (0.885) AL length (0.669)
ANOVA			
P_{CO_2}	$F_{1,229}$=4.78, ***P*=0.03**	$F_{3,229}$=2.96, P=0.08	$F_{3,229}$=1.67, P=0.197
Female	$F_{3,229}$=1.89, P=0.13	$F_{3,229}$=0.78, P=0.51	$F_{3,229}$=2.06, P=0.11
P_{CO_2} × female	$F_{3,229}$=7.30, ***P*<0.001**	$F_{3,229}$=2.18, P=0.09	$F_{3,229}$=1.30, P=0.27
Six-arm larvae			
% of variance	42.49	19.02	10.81
Cumulative % of variance	42.49	61.51	72.33
Eigenvalue	5.10	2.28	1.30
Highest loading factors (component score)	Body width (0.904) Distance between PO (0.873) Body length:width ratio (0.868)	Stomach volume (0.941) Stomach length (0.864) Stomach width (0.765)	AL length (0.861) PD length (0.799)
ANOVA			
P_{CO_2}	$F_{1,233}$=63.3, ***P*<0.001**	$F_{3,233}$=7.90, ***P*<0.001**	$F_{3,233}$=4.79, ***P*=0.003**
Female	$F_{3,233}$=7.56, ***P*=0.006**	$F_{3,233}$=5.04, ***P*=0.002**	$F_{3,233}$=7.32, ***P*<0.001**
P_{CO_2} × female	$F_{3,233}$=6.95, ***P*=0.009**	$F_{3,233}$=8.61, ***P*<0.001**	$F_{3,233}$=3.26, ***P*=0.022**
Eight-arm larvae			
% of variance	49.464	13.699	9.803
Cumulative % of variance	49.464	63.163	72.966
Eigenvalue	6.925	1.918	1.372
Highest loading factors (component score)	Distance between PD, AL, PO (0.80,0.77,0.64) PD, PO, AL length (0.85,0.81,0.80) Body width (0.61)	Stomach volume (0.94) Stomach width (0.91) Stomach length (0.82)	Body length (0.79) Body length:width ratio (–0.83)
ANOVA			
P_{CO_2}	$F_{1,232}$=1.78, P=0.67	$F_{3,232}$=107.10, ***P*<0.001**	$F_{3,232}$=2.63, P=0.11
Female	$F_{3,232}$=6.16, ***P*<0.001**	$F_{3,232}$=18.98, ***P*<0.001**	$F_{3,232}$=4.75, ***P*=0.003**
P_{CO_2} × female	$F_{3,232}$=16.04, ***P*<0.001**	$F_{3,232}$=2.81, ***P*=0.04**	$F_{3,232}$=11.40, ***P*<0.001**

Because morphological characteristics measured are correlated, principal component analysis (PCA) was used for data reduction. Rotated component matrix after varimax rotation with Kaiser normalization was applied to this PCA. Statistical significance of the effects of elevated P_{CO_2}, maternal lineage and their interactions on the component scores were tested with two-way ANOVA. Bold *P*-values indicate a significant difference (P<0.05). AL, anterolateral arm; PD, posterodorsal arm; PO, postoral arm; PR, preoral arm.

in anterolateral arm lengths and in distances between tips of posterodorsal and preoral arms. Maternal lineage had significant effects on all three PCA component scores: (1) larval body widths were wider and arm lengths were longer in offspring of female 4 than in offspring of females 1 and 3 (PCA axis 1 scores, P=0.001; (2) offspring of female 4 had the largest stomach, and offspring of female 2 had larger stomachs than offspring of females 1 and 3 (PCA axis 2 scores, P<0.001); and (3) offspring of females 1 and 3 had longer body lengths than offspring of female 4 (PCA axis 3 scores, P=0.003). There were significant interactive effects of CO_2 treatment and maternal lineage on larval body width (PCA axis 1 scores, P<0.001), stomach size (PCA axis 2 scores, P=0.04) and larval body length (PCA axis 3 scores, P<0.001).

Swimming performance analysis

Larval developmental stage had a significant effect on total speed, oscillatory speed and net vertical and horizontal velocities of both up- and down-swimming individuals, such that swimming speeds increased with larval stage (Fig. 3, supplementary material Table S1; $F_{1,129}$≥5.32, P≤0.02). Developmental stage also affected the shape of swimming trajectories for up-swimming individuals: older larvae swam in wider helices ($F_{1,129}$=4.32, P=0.04; supplementary material Table S1). However, CO_2 treatment alone did not have a significant impact on any swimming metrics. Maternal lineage significantly affected net horizontal velocity ($F_{3,129}$=4.32, P=0.01) and helical pitch ($F_{3,129}$=7.12, P<0.001) among up-swimming individuals, but there was no significant maternal effect on any swimming metrics of down-swimming individuals. There were no significant interaction effects between CO_2 treatment and maternal lineage on any swimming metrics (Fig. 2, supplementary material Table S1).

Short-term changes in the P_{CO_2} level in the surrounding water had no effect on swimming speeds or helical parameters (Table 3). Maternal lineage had a significant effect on the helical geometry of the swimming trajectories of down-swimming individuals, both in pitch (P=0.02) and width (P<0.001), despite there being no significant morphological differences between the two observed lineages. There were no significant interaction effects between CO_2 treatment and maternal lineage on any swimming metrics (Table 3).

DISCUSSION

OA negatively affects many calcifying organisms, including many planktonic marine invertebrate larvae that are believed to be highly vulnerable to environmental changes (Kurihara, 2008). In our experiments, growth under acidified conditions induced

Table 3. Swimming metrics (means ± s.e.m.) of four-arm larval *Dendraster excentricus* reared under present-day conditions exposed to short-term change in ambient CO_2

	P_{CO_2} of surrounding water			
	380 ppm		1000 ppm	
	Female 3	Female 4	Female 3	Female 4
Up-swimmers				
Total speed ($\mu m\,s^{-1}$)	256.80±27.10	289.59±16.43	282.46±4.67	309.54±21.33
Oscillating speed ($\mu m\,s^{-1}$)	246.71±25.43	276.27±15.26	269.45±14.35	294.33±19.61
Net horizontal speed ($\mu m\,s^{-1}$)	33.24±2.80	46.56±6.66	31.78±23.51	31.22±4.61
Net vertical speed ($\mu m\,s^{-1}$)	119.67±19.53	146.87±12.00	147.60±22.02	166.15±16.24
Helical pitch (mm)	1.61±0.48	2.14±0.76	1.78±0.35	2.00±0.44
Helical width (mm)	0.40±0.06	0.34±0.04	0.40±0.04	0.34±0.04
Down-swimmers				
Total speed ($\mu m\,s^{-1}$)	259.22±22.97	256.39±24.59	185.70±34.35	241.76±20.46
Oscillating speed ($\mu m\,s^{-1}$)	248.16±21.12	245.05±22.71	176.99±31.81	230.06±19.00
Net horizontal speed ($\mu m\,s^{-1}$)	30.30±6.71	31.26±4.34	29.07±7.48	47.92±10.83
Net vertical speed ($\mu m\,s^{-1}$)	−127.9±19.00	−128.38±18.21	−93.91±24.15	−124.60±14.85
Helical pitch (mm)	0.45±0.13	0.93±0.23	0.24±0.08	0.99±0.22
Helical width (mm)	0.26±0.09	0.37±0.03	0.11±0.03	0.24±0.04

morphological changes in larval *D. excentricus*: when reared in a P_{CO_2} concentration of 1000 ppm, larvae had narrower bodies and smaller stomachs and, in the last observed stage, shorter arms. Previous modeling and experimental observations have suggested that such changes in larval morphology are likely to alter swimming performance (Clay and Grünbaum, 2010). However, the morphological changes observed in our study were not associated with negative impacts on the measured swimming performance metrics. There were also no observable changes in swimming metrics during short-term (1 h) exposure to changes in ambient P_{CO_2} concentration. Both our video observations and hydrodynamic modeling suggest that the morphological changes were coordinated to preserve swimming performance, an ecologically important function. However, the fact that acidified larvae in our study had smaller stomachs, coupled with previous observations that stomach size reflects larval nutrition, suggests that the negative effects of OA on early stages were carried over to later stages.

OA-induced morphological changes may compensate for stability loss

The morphological effects we observed are consistent with previous studies in which OA negatively impacted growth and physiology of other larval urchins (Kurihara, 2008; Clark et al., 2009; Todgham and Hofmann, 2009; O'Donnell et al., 2010). However, those studies on larval urchins were short (2–4 days) and the larvae were not fed. We monitored growth of fed larval sand dollars for 10 days, over 60% of their approximately 14–16 day larval duration at 20°C (Strathmann, 1987). Our observations showed consistent differences in larval morphologies between pH treatments at all three developmental stages. Though feeding provided additional resources to potentially compensate for OA impacts, the acidified larval sand dollars we observed remained smaller throughout their development than their siblings reared under present-day P_{CO_2} conditions (Fig. 2, Table 2).

At the high P_{CO_2} level, larval urchins showed significantly reduced calcification (Clark et al., 2009). Furthermore, our acidified sand dollars were smaller and showed no sign of increased skeletal rod thickness or decreased fenestration under polarized light (K.Y.K.C., personal observation), suggesting an overall reduction in calcification. A number of mechanisms could potentially have led to reduced calcification under OA, including decreased carbonate ion availability, increased metabolic cost to deposit calcite and increased dissolution rate (Doney et al., 2009). Regardless of the underlying mechanisms, one implication of reduced calcification is that, under acidification, the total amount of skeletal material that an individual can possess is limited.

Smaller size and reduced calcification has at least two biomechanical consequences for larval swimming performance. First, hydrodynamic models indicate that isometric shrinking of 'armed larvae' reduces their stability (Grünbaum and Strathmann, 2003). Second, because larval weight distribution is strongly dependent on calcite, reduced calcification (e.g. reduction or elimination of counter-weights) further compromises larval stability (Pennington and Strathmann, 1990). Hence, through both mechanisms, a likely consequence of OA is a decreased ability to maintain oriented, directional swimming in moving water.

Hydrodynamic models suggest two hypothetical morphological adjustments that could at least partially restore stability to larvae with limited calcification (Grünbaum and Strathmann, 2003). The first possible adjustment is shortening skeletal arm rods, which would reduce the destabilizing torque imposed on larvae by shear flows. The second possible adjustment is increasing arm elevation angles, which would increase the stabilizing torque by increasing the separation distance between the centers of buoyancy and gravity. The first of these adjustments is likely to negatively impact feeding, because particle capture rate is dependent on the total length of the ciliated band around the larval arms. In contrast, there is no known strong relationship between feeding performance and the second adjustment, i.e. increasing arm elevation angles (though other swimming performance metrics, such as weight-bearing capacity, may be compromised). If both swimming and feeding performance are to be preserved under OA, the trade-off between swimming stability and feeding efficiency suggests that growth leading to high arm elevation angles would be favored, and shortening of arms would be disfavored.

The four-, six- and eight-arm acidified larvae we observed had narrower bodies than those reared under present-day conditions. There was no significant difference in arm lengths or body heights between pH treatments at the four- and six-arm stages. These observed morphological changes are consistent with the second hypothetical

morphological adjustment to increase stability. Arm elevation angles in plutei are determined by the attachment points of transverse body rods, determined by body width and height, and by the lengths of arm rods (Fig. 2). Shortening transverse rods without commensurate movement of their attachment points narrows the larval body, elevates larval arms to increased angles and hence increases stability in shear.

The eight-arm acidified larvae we observed, in addition to narrower bodies, also had shorter larval arms than their siblings reared under present-day conditions. A possible explanation may be that limitation on calcification became so extreme at this larval stage that preserving arm lengths was no longer physiologically possible. Because larval sand dollars develop juvenile rudiments at

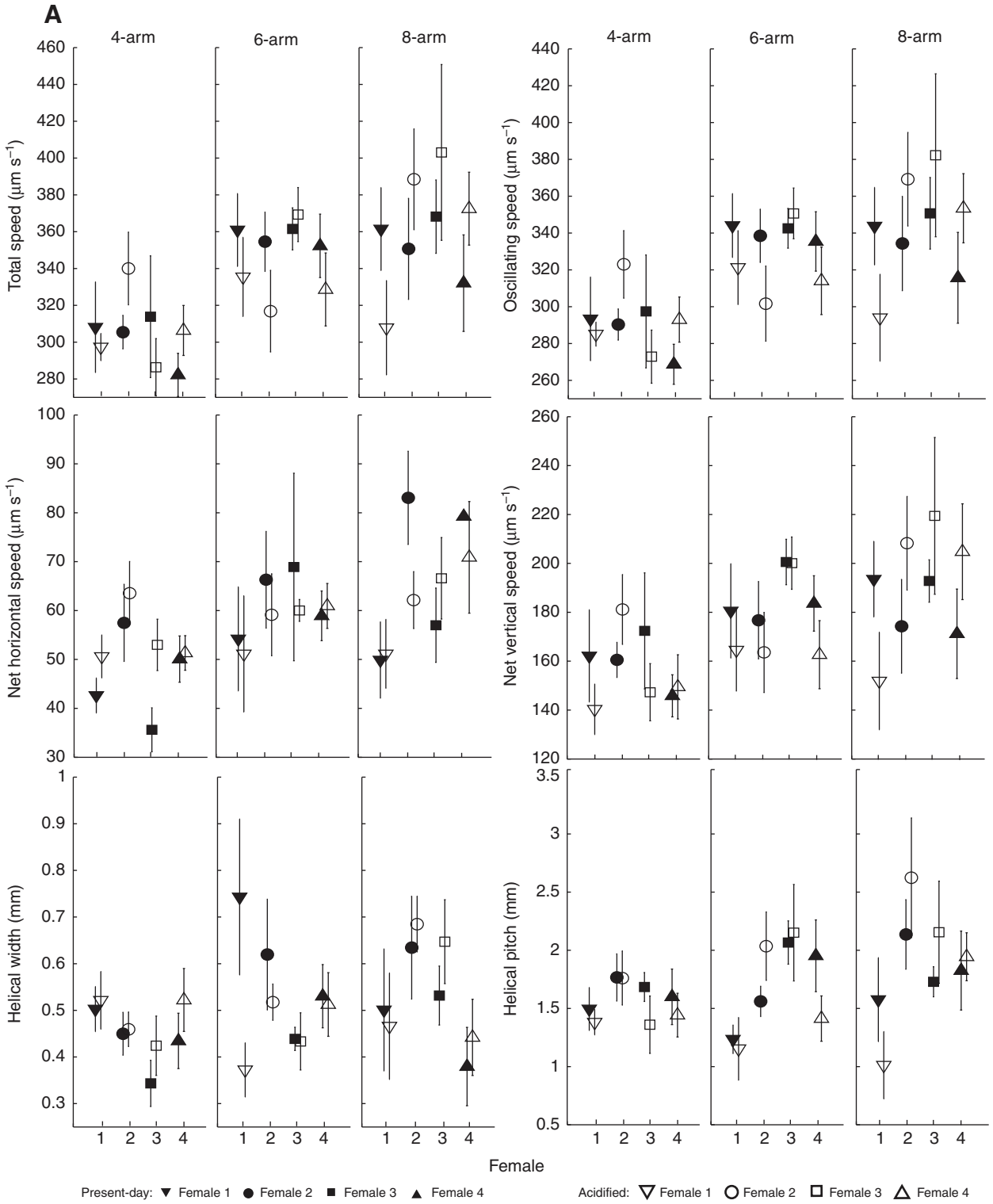

Fig. 3. Continued on next page.

the eight-arm stage, it is also possible that acidified eight-arm larvae had shorter arms because the energy provided by feeding could not support concurrent growth of larval body and rudiment, or that the relative payoff for investment in juvenile structures becomes more favorable at this stage than investment in a larval body that will soon be discarded.

Swimming performance was maintained despite morphological changes

In contrast to stage-dependent morphological changes, which are known to impact swimming (Clay and Grünbaum, 2010), the OA-induced morphological changes in our experiments were not associated with significant effects on larval swimming performance

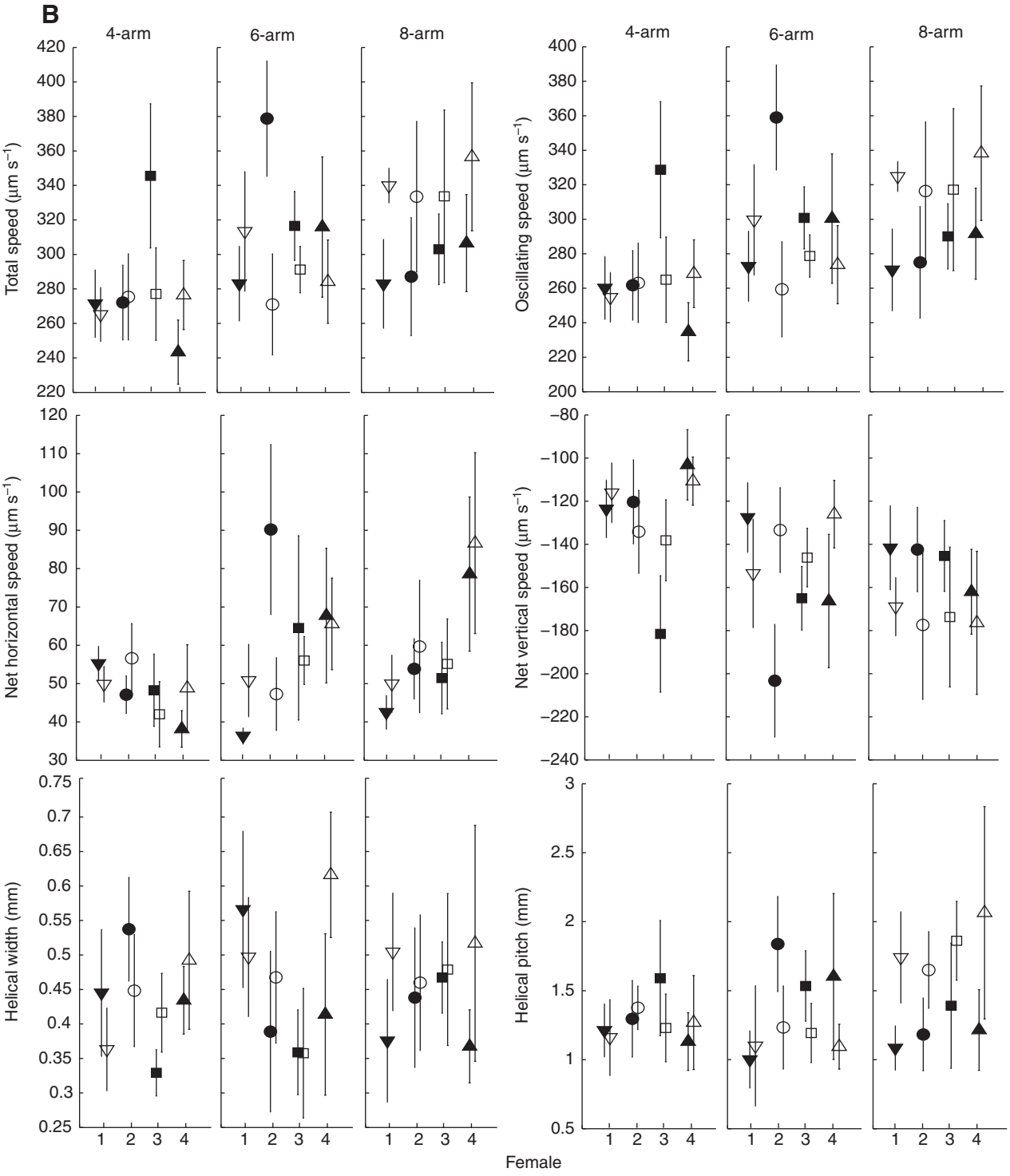

Fig. 3. Swimming metrics (means ± s.e.m.) of larval *D. excentricus* in acidified and present-day treatments at four-, six- and eight-arm stages: (A) net up-swimming and (B) net down-swimming. Symbols denote different maternal lineages. Closed symbols indicate larvae in the present-day treatment (380 ppm) and open symbols indicate larvae in the acidified treatment (1000 ppm).

metrics (total speed, oscillatory speed and net vertical velocity; Fig. 3, supplementary material Table S1).

In addition to coordinated morphological changes, another possible mechanism through which larval sand dollars may have maintained consistent swimming is by modulating ciliary beat patterns. Reversals in ciliary beat during particle capture have been described in detail (Strathmann, 1975), but more subtle shifts such as those potentially involved in swimming adjustments have not been explored. Apparent behavioral compensation for temperature decreases that maintained vertical swimming speeds despite reductions in total swimming speed has been observed amongst larval sand dollars (Chan and Grünbaum, 2010). This compensation was reflected in reduced widths of helical trajectories, which had the effect of reducing the total distance traveled for a given vertical excursion. Because changes in trajectory characteristics occurred over short time scales (minutes), morphological adjustments are a less likely mechanism for compensation than rapid behavioral adjustments such as shifts in ciliary beat.

Impact of OA carried over to later stages by reducing feeding performance

Our acidified larvae, though fed, had reduced stomach sizes. Several possible explanations may account for this observation. Each of these interpretations suggests that OA negatively impacts larval nutrition.

One possible explanation for smaller stomachs for acidified larvae is that acidification affects ciliary beat pattern and reduces particle capture efficiency. Ciliary reversals, and hence particle capture, in echinoplueti are associated with the opening and closing of calcium ion channels (Hart, 1990). It is possible that changes in pH affect ionic exchanges such that ciliary reversal and hence particle captures occur less frequently. Consistent with this hypothesis, the number of algal particles ingested by acidified larval purple sea urchins decreased with a 0.4 unit drop in pH (Stumpp et al., 2011). A likely byproduct of fewer ciliary reversals is more propulsive force generated per unit time. If this interpretation is true, it opens up a promising avenue of research on the trade-off between feeding and swimming at the ciliary level.

A second hypothesis for small larval stomach size in acidified larvae is that acidification elevates larval metabolism, increasing the digestion rate and therefore decreasing stomach distention. If so, an implication is that the elevated metabolic demands of acidified larvae were not met in our experiment. This is consistent with smaller overall sizes we observed in acidified larvae. A third hypothesis is that larval sand dollar may be responding to OA stress in a way similar to starvation, i.e. by growing longer arms and smaller stomachs. However, in contrast to known starvation response, observed arm length of acidified larvae were not significantly longer than their siblings across all stages, suggesting that larvae were not able to compensate for food limitation stress by growing longer arms.

Regardless of the underlying mechanism causing the observed smaller stomach sizes, our data and that of other studies consistently suggest that acidification has negative impacts on larval nutrition. It has long been established that larval nutritional conditions have significant impacts on the success of post-settlement stages (Pechenik et al., 1998). These impacts suggest that the negative effects of OA on feeding, though sublethal in the laboratory at early larval stages, are likely to carry over to subsequent developmental stages and hence have significant population-level consequences under natural conditions.

Putting our experimental observations into an ecological context, our experimental sand dollars were collected in East Sound, a shallow embayment in Washington, USA. Intertidal areas experience large diurnal fluctuations in pH (Wootton et al., 2008) and in upwelling areas such as the Washington coast, pH of the surface water is influenced by acidic deep waters (Feely et al., 2008). It is therefore possible that sand dollars are acclimated to variations in pH level, at least within our experimental range. In the face of an apparent trade-off between swimming and feeding performance, preserving swimming over feeding performance under acidified conditions might have been less deleterious than its converse. Larval sand dollars are known to uptake dissolved organic matter (Davis and Stephens, 1984) and complete metamorphosis even under low food concentrations (Boidron-Metairon, 1988). This ability to cope with low feeding rates suggests that the immediate negative consequences of failure to swim (e.g. an inability to avoid predators and/or locate settlement sites) may outweigh the long-term cost of smaller juveniles. If that were the case, larval morphologies that result in good swimmers but poor eaters could be beneficial for larval survival (Strathmann and Grünbaum, 2006).

Sensitivity to OA varied between families within a population

Both maternal lineage and its interaction with CO_2 treatment had significant effects on larval morphology (Tables 1, 2), suggesting that there is among-family variability in sensitivity to changes in P_{CO_2}. The differences we observed between offspring of different females probably did not result from variations in maternal provision, because egg size did not differ between females and because larvae were fed throughout the experiment (Bertram and Strathmann, 1998). Byrne et al. (Byrne et al., 2010) hypothesized that there is substantial within-population variation in OA sensitivity, and also suggested that polyandry *versus* single male–female crossing could explain the contradiction between their results and those of Havenhand et al. (Havenhand et al., 2008) concerning effects of OA on fertilization success in the sea urchin *Heliocidaris erythrogramma*. Our results show that there is indeed substantial within-population variation in OA sensitivity, at least in sand dollars. For example, had we made observations only on offspring of female 4 at the eight-arm stage, we would have concluded that acidified larvae were larger and had a higher total swimming speed. However, our conclusion would have been the opposite had we observed only offspring of female 1 (Table 1, Fig. 3). Variation of this kind reinforces the importance of including multiple parental lineages in future OA experiments, to reduce variability between experimental runs (Evans and Marshall, 2005), make population-level generalizations more reliable and better predict community-level responses to changing environments.

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