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Development Under Elevated pCO_2 Conditions Does Not Affect Lipid Utilization and Protein Content in Early Life-History Stages of the Purple Sea Urchin, *Strongylocentrotus purpuratus*

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Abstract. Ocean acidification (OA) is expected to have a major impact on marine species, particularly during early life-history stages. These effects appear to be species-specific and may include reduced survival, altered morphology, and depressed metabolism. However, less information is available regarding the bioenergetics of development under elevated CO_2 conditions. We examined the biochemical and morphological responses of *Strongylocentrotus purpuratus* during early development under ecologically relevant levels of pCO_2 (365, 1030, and 1450 μatm) that may occur during intense upwelling events. The principal findings of this study were (1) lipid utilization rates and protein content in *S. purpuratus* did not vary with pCO_2; (2) larval growth was reduced at elevated pCO_2 despite similar rates of energy utilization; and (3) relationships between egg phospholipid content and larval length were found under control but not high pCO_2 conditions. These results suggest that this species may either prioritize endogenous energy toward development and physiological function at the expense of growth, or that reduced larval length may be strictly due to higher costs of growth under OA conditions. This study highlights the need to further expand our knowledge of the physiological mechanisms involved in OA response in order to better understand how present populations may respond to global environmental change.

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Abbreviations: ELS, early life-history stages; hpf, hours post fertilization; OA, ocean acidification; PL, phospholipid; PO, postoral; ST, sterol; TG, triglyceride.

Introduction

Ocean acidification—the decrease of ocean pH *via* absorption of anthropogenic CO_2 by surface waters—has emerged as a major priority in the marine science research community (*e.g.,* National Research Council, 2010). In subdisciplines from chemical oceanography to invertebrate zoology, investigators are working to forecast the effects of future acidification from the organismal to the ecosystem level. To date, the primary focus of organismal research has centered on the impacts of altered seawater chemistry on calcifying marine organisms—those that use biogenic calcification to form a calcium carbonate shell or skeleton (reviewed by Andersson *et al.,* 2008; Cohen *et al.,* 2009; Hofmann *et al.,* 2010). As investigations on organismal performance have developed, much of the focus has centered on early life-history stages (ELS) (Byrne, 2011, 2012), largely due to the fact that many ELS produce calcified structures and they represent an important bottleneck to population persistence due to their potentially high vulnerability to environmental stress (Thorson, 1950; Rumrill, 1990; Morgan, 1995; Melzner *et al.,* 2009; Byrne, 2012).

Research focusing on the costs of ELS developing under OA conditions has assessed physiological processes beyond calcification, including studies on fertilization (Havenhand *et al.,* 2008; Byrne *et al.,* 2010; Parker *et al.,* 2010), survival (Dupont *et al.,* 2008; Talmadge and Gobler, 2010), growth and morphology (Kurihara *et al.,* 2009; Gazeau *et al.,* 2010;

Dupont *et al.,* 2010a; Sheppard Brennand *et al.,* 2010; Yu *et al.,* 2011), metabolism (Albright and Langdon, 2011; Stumpp *et al.*, 2011a), patterns of gene expression (Todgham and Hofmann, 2009; O'Donnell *et al.,* 2010; Martin *et al.,* 2011; Stumpp *et al.*, 2011b; Evans *et al.*, in press), and behavior (Ellis *et al.,* 2009; Dixson *et al.,* 2010). Thus far, species have shown both positive and negative responses to OA, though meta-analyses indicate that on average the response is negative (Dupont *et al.,* 2010b; Kroecker *et al.,* 2010). Additionally, research on juvenile corals and adult mussels suggests that calcium carbonate deposition under OA incurs a substantial energetic cost (Cohen *et al.*, 2009; Melzner *et al.*, 2011). Up to this point there has been a deficit of studies exploring the role of metabolism and energy utilization during development under OA (for recent work see Albright and Langdon, 2011; Nakamura *et al.,* 2011; Stumpp *et al.,* 2011a, 2011b; Wong *et al.*, 2011; Dineshram *et al.*, 2012). Understanding how biochemical resources are utilized during early development, particularly lipid and protein, may be a fruitful avenue to further explore the physiological costs and responses to OA.

Maternal provisioning of energetic resources is important to the development of ELS. There is a rich literature examining the maternal variation in egg investment and its influence on offspring growth and fitness (reviewed by Marshall *et al.,* 2008). McEdward (1986) found that across echinoid species, larger eggs produced larger larvae, presumably due to greater resource provisions. In strongylocentrotid sea urchins, experimental evidence suggests that female diet has little effect on larval size and developmental timing (Bertram and Strathmann, 1998; Meidel *et al.,* 1999). Work by Sunday *et al.* (2011) has shown that a strong maternal component does exist, though their experiments did not address diet. Lipids are the predominant form of endogenous energy that fuels larval development in a number of marine taxa (Holland, 1978), and along with protein composes about 80% of the available energy within an egg (Jaeckle, 1995). In invertebrates with planktotrophic larvae, eggs are provisioned with sufficient energy for maintaining physiological demands and building the initial larval body but are dependent on exogenous resources for further development; in contrast, lecithotrophic larvae are provisioned with greater levels of endogenous resources to fuel both larval and juvenile development without feeding (Jaeckle, 1995). Triglyceride (or triacylglyceride) has been identified as the primary energy storage lipid during early development in echinoderms, with high initial abundances metabolized during pre-feeding development (Yasumasu *et al.,* 1984; Sewell, 2005; Meyer *et al.,* 2007; Byrne *et al.,* 2008; Prowse *et al.,* 2008). Reductions in energy resources available to ELS may result in smaller or slower developing larvae (Sinervo and McEdward, 1988; Emlet and Hoegh-Guldberg, 1997; Allen *et al.,* 2006), which could have negative consequences such as increasing the time in which larvae are exposed to predation in the water column (Allen, 2008) and could ultimately lead to lower rates of benthic recruitment (Morgan, 1995).

Understanding the pattern of energetic costs during development under OA conditions may shed further light on how ELS respond to OA. Directional changes in the abundance of endogenous resources, as well as the magnitude of change, may indicate whether OA conditions are indeed stressful to developing organisms. In adult metazoans, physiological responses to environmental stress are known to incur large energetic costs (Feder and Hofmann, 1999; Hochachka and Somero, 2002), a situation that might be expected in ELS. However, OA-induced reduction in metabolic activity is common in CO_2-sensitive marine invertebrates and expected to be more pronounced in ELS (see Melzner *et al.,* 2009; Albright and Langdon, 2011). Previous work with larval sea urchins has found OA stress-responsive patterns of gene expression that suggest metabolic depression (Todgham and Hofmann, 2009; O'Donnell *et al.,* 2010; but see Stumpp *et al.,* 2011a). While reducing metabolic demand may be useful during short periods of unfavorable environmental conditions, prolonged depression could lead to reductions in growth and development (Guppy and Withers, 1999). Thus, quantification of endogenous energy utilization during development in response to altered seawater chemistry and pH may serve as a proxy for metabolic activity, and function as a window into the energetic cost of OA. Such an approach has been used extensively in the non-OA literature, primarily focused on starvation effects in echinoderm larvae (*e.g.,* Shilling and Manahan, 1990, 1994; Marsh *et al.,* 1999; Sewell, 2005; Meyer *et al.,* 2007). Echinoid larvae have been shown to be quite tolerant of starvation during early development, with studies demonstrating survival of unfed larvae over periods of 2 weeks or more (Sewell, 2005; Pace and Manahan, 2006; Meyer *et al.,* 2007), 4 weeks or more (Eckert, 1995; Moran and Allen, 2007; Yu, 2009), and 8 weeks or more (Marsh *et al.,* 1999). During this period without food, larval echinoids have previously been shown to develop longer postoral arms (Boidron-Metairon, 1988; Hart and Strathmann, 1994; McEdward and Herrera, 1999; Sewell *et al.,* 2004; Miner, 2007; Adams *et al.*, 2011). This additional period of growth may result in larger skeletons with which to detect pCO_2-dependent differences in larval size, though care should be taken when comparing to studies with larvae that were fed.

In this study, we measured endogenous lipid and protein during early development of sea urchin larvae reared under OA conditions—low pH and decreased saturation states for calcium carbonate species ($\Omega_{Aragonite}$ and $\Omega_{Calcite}$). Our study species, *Strongylocentrotus purpuratus* (Stimpson, 1857), the purple sea urchin, is a conspicuous and important member of rocky subtidal marine communities along the

western coast of North America. This species is often found in regions with strong seasonal upwelling, which has been positively linked to low pH events (pH and pCO_2 levels ranging from 8.1 to 7.6 and 350 to 1100 μatm; Feely *et al.,* 2008) and may cause it to experience large fluctuations in pH over short time scales (Yu *et al.,* 2011; Hofmann *et al.*, 2011). When raised under elevated pCO_2 conditions (1444 *vs.* 365 μatm), unfed *S. purpuratus* plutei experienced a significant decrease in total length at 72 and 144 h post fertilization (hpf), but the authors (Yu *et al.,* 2011) could not determine whether this reduced size was due to slower growth (potentially *via* metabolic depression and/or increased costs of calcification) or developmental delay. The work presented here is a companion study to Yu *et al.* (2011), using samples collected from the same experimental treatments. Lipid utilization rates have been shown to respond to physiologically stressful conditions in other marine species (*e.g.,* Montero *et al.,* 1999; Torres *et al.,* 2002; Moran and Manahan, 2004), but this response has yet to be investigated in echinoid larvae within the context of OA. Thus, we might predict that if growth and maintenance under high CO_2 induces an increased energetic cost, relative differences in lipid resources will be greater in elevated pCO_2 treatments than in controls. Alternatively, if *S. purpuratus* is adapted to environmental heterogeneity, it may have sufficient physiological plasticity to develop successfully under variable pH conditions without a major change in energy resource utilization.

Materials and Methods

Larval culturing

Gravid adult purple urchins (*Strongylocentrotus purpuratus*) were collected by scuba divers off Goleta Pier (34°24′57.84″N, 119°49′43.96″W) at a depth of 5.5 m in November 2009, and maintained in flowing seawater aquaria (ambient temperature of about 15–16 °C) at the University of California, Santa Barbara until they spawned. Adult urchins were fed *ad libitum* with *Macrocystis pyrifera* until they were used in experiments.

Gametes were obtained by coelomic injection of 0.5 mol l^{-1} KCl. Eggs were suspended in 0.35-μm-filtered, UV-sterilized seawater (FSW) at ambient temperature and pCO_2 for fertilization. Sperm was collected dry and then diluted in FSW at ambient temperature and pCO_2 immediately prior to fertilizing a batch of eggs. Sperm from one male was used to fertilize the eggs of four females. Fertilization success for all batches of eggs was greater than 90%. Fertilized embryos were counted and then immediately stocked into larval culture vessels at 300,000 per chamber (~20 embryos ml^{-1}). Fertilized embryos from each female were split across all pCO_2 treatments, resulting in four culture vessels per treatment ($n = 4$). Each culture vessel had a constant volume of 15 l (additional details below.)

We allowed embryos to develop for 144 h (6 days) to the 4-arm pluteus stage. Although larvae held at these temperatures are capable of feeding at about 70 hpf (Strathmann, 1987), we intentionally withheld any food subsidies in order to (1) force the larvae to utilize only their endogenous lipid resources to fuel development, and (2) allow larvae time to develop a larger skeleton. We chose to allow development to continue until day-6 without food subsidy on the basis of lipid data presented by Meyer *et al.* (2007) and Adams *et al.* (2011), in which total lipid content was 15%–20% of the initial levels present in the egg.

Eggs were sampled prior to fertilization; embryos were sampled at mid-gastrula stage (25–26.5 hpf); and larvae were sampled at day-3 (72 hpf) and day-6 (144 hpf). Egg samples consisted of 2000 and 500 eggs per sample (lipid: $n = 3$; protein: $n = 4$, respectively); embryo and larval samples consisted of 1000 and 500 individuals each (lipid: $n = 3$; protein: $n = 4$, respectively). Larvae were immediately concentrated by reverse siphoning, counted volumetrically, and then distributed to sample tubes for fixation (morphology) or for centrifugation into a pellet for immediate freezing and storage at −80 °C (biochemistry).

CO_2 mixing system

The CO_2 gas mixing system as described in Fangue *et al.* (2010) was used to manipulate pCO_2 levels in larval culture chambers. Briefly, this system uses mass flow control valves to mix CO_2-free air with pure CO_2 gas, which is then bubbled into seawater reservoirs until a desired equilibrium is reached. From these reservoirs, treated seawater is pumped to larval culture chambers (15 l) at a rate of 1 $l \cdot h^{-1}$, allowing flow-through culturing of larvae. Chambers are kept continuously mixed using an acrylic paddle attached to an electric motor. A minor modification to the reported design of the larval culture chamber was added to maintain high levels of pCO_2 in seawater: the gas mixture used to equilibrate the seawater in the reaction vessels was also simultaneously pumped into the headspace of the larval culture chambers, keeping the headspace pCO_2 at the same concentration as the treated water. Larvae were removed from vessels by siphoning to minimize the disturbance of vessel lids and prevent gas exchange in the headspace.

Seawater chemistry

Total alkalinity, pH, temperature, and salinity were measured on the first day of the experiment prior to fertilization, and subsequently at each sampling interval ($t = 0$, 25 hpf, 72 hpf, and 144 hpf); the measured parameters are shown in Table 1. Temperature in the culture chambers (15.6 ± 1.1 °C) was measured using a calibrated wire-thermocouple (Thermolyne PM 20700/Series 1218). Salinity of incoming seawater (33.1–33.2) was measured using a bench-top digital salinity meter (YSI 3100 conductivity). Methods for CO_2

Table 1

Carbonate parameters in the larval culture vessels

pCO_2	Initial	Gastrula (25 h)	4-arm pluteus (72 h, day-3)	4-arm pluteus (144 h, day-6)
pH				
365 μatm	8.06 ± 0.03	8.07 ± 0.02	8.07 ± 0.00	8.07 ± 0.00
1030 μatm	7.63 ± 0.03	7.68 ± 0.00	7.67 ± 0.00	7.68 ± 0.00
1450 μatm	7.52 ± 0.02	7.54 ± 0.01	7.53 ± 0.00	7.54 ± 0.01
TA ($\mu mol \cdot kg^{-1}$)				
365 μatm	2214.3	2211.4	2213.1	2223.3
1030 μatm	2222.9	2214.9	2209.8	2224.3
1450 μatm	2216.3	2216.5	2208.6	2214.6
pCO_2 (μatm)				
365 μatm	373.2 ± 26.2	361.3 ± 15.1	363.8 ± 2.3	363.2 ± 3.0
1030 μatm	1125.7 ± 85.8	997.4 ± 9.5	1015.9 ± 12.4	1016.7 ± 9.3
1450 μatm	1492.9 ± 86.2	1422.9 ± 18.2	1431.3 ± 15.9	1429.4 ± 35.1
$\Omega_{aragonite}$				
365 μatm	2.38 ± 0.12	2.42 ± 0.07	2.43 ± 0.02	2.46 ± 0.02
1030 μatm	1.00 ± 0.07	1.10 ± 0.01	1.09 ± 0.01	1.10 ± 0.01
1450 μatm	0.77 ± 0.04	0.81 ± 0.01	0.80 ± 0.01	0.81 ± 0.01
$\Omega_{calcite}$				
365 μatm	3.72 ± 0.19	3.78 ± 0.10	3.79 ± 0.02	3.84 ± 0.03
1030 μatm	1.56 ± 0.11	1.71 ± 0.01	1.69 ± 0.02	1.72 ± 0.01
1450 μatm	1.21 ± 0.06	1.26 ± 0.01	1.26 ± 0.01	1.27 ± 0.02

Total alkalinity (TA) and pH (total scale) were the measured parameters; the remaining parameters were calculated using CO2SYS. Mean and standard deviation across larval culture vessels are shown at each sampling time point.

analysis, as modified from Standard Operating Procedures (SOP) for pH (SOP6b) and Total Alkalinity (TA, SOP 3b) (Dickson *et al.,* 2007), are reported in Fangue *et al.* (2010). All pH values use the total scale. All other carbonate parameters were calculated in CO2SYS, ver. 1.0, for MS Excel (Pierrot *et al.,* 2006) using the constants of Mehrbach *et al.* (1973) as refit by Dickson and Millero (1987).

Lipid extraction and quantification

Lipid was extracted from frozen egg, embryo, and larval samples following the method of Holland and Gabbott (1971) and modified by Sewell (2005). Sample homogenates were spiked with 25 μl of internal standard (3-hexadecanone, 1 $\mu g \cdot \mu l^{-1}$ in chloroform) and prepared so that the final mixture had a ratio of water/chloroform/methanol of 2:1:2 (Bligh and Dyer, 1959). The ketone internal standard provides an estimate of lipid recovery in the final concentrate (Parrish, 1987) as it is primarily a vertebrate lipid class and, if present, is only in very low concentrations in marine invertebrate tissue. Phases were separated by adding chloroform and water so that the final ratio of water/chloroform/methanol was 4:3:2 (v:v:v). Following centrifugation at 1000 × *g* for 5 min, the lower chloroform layer was removed, dried down under a stream of N_2 gas, and suspended in 25 μl of chloroform immediately prior to spotting.

The amount of each lipid class in the lipid extract was quantified using an Iatroscan Mark V^{new} thin layer chromatography/flame ionization detection system and silica gel S-III Chromarods following the protocols established by Parrish (1999) and modified by Sewell (2005). To each rod, 5 μl of sample was added using a microdispenser pipette (Drummond Scientific). Chromarods were developed in a double development system modified from Parrish (1999), which produced two separate chromatograms. The rod rack was first developed in a 98.95:1:0.05 (v:v:v) solution of hexane/diethylether/formic acid for 25 min. Chromarods were partially scanned and then redeveloped in a 79:20:1 (v:v:v) solution of hexane/diethylether/formic acid for 36 min. The rod rack was then returned to the drying oven for 5 min prior to the final full scan. Data were collected with an SES-Chromstar PC-board, and the peaks were quantified using SES-Chromstar ver. 4.10 (SES Analysesysteme).

Lipid quantification was based on multilevel calibration curves generated for each lipid class using a five-component composite standard made from highly purified lipid standards in HPLC-grade chloroform. The lipid classes were ketone (KET; 3-hexadecanone), aliphatic hydrocarbon (Nonadecane), triglyceride (TG; Tripalmitin), free fatty acid (palmitic acid), sterol (ST; cholesterol), and phospholipid (PL; L-α-phosphatidylcholine). Lipid abundance was corrected to 100% recovery by comparison with the internal standard (KET). Two lipid classes were not used in quantification of the energetic costs of sea urchin larvae: aliphatic hydrocarbon, which was used primarily as a metric of

chloroform contamination, and free fatty acid, which showed levels routinely at or below the level of detection for this method. Total lipid is therefore represented by the sum of the remaining lipid classes (triglyceride, sterol, and phospholipid).

Determination of protein content

Frozen eggs, embryos, and larvae were suspended in cold MilliQ water and sonicated. Total protein content was determined using a Bradford assay as modified for invertebrate larvae (Jaeckle and Manahan, 1989). Briefly, total protein from larval homogenates was precipitated in 5% trichloroacetic acid (TCA) at 4 °C, and centrifuged at 14000 × *g*. This pellet was washed with additional TCA, and solubilized in 1 mol l^{-1} NaOH at 56 °C. The solubilized protein was neutralized with 1.68 mol l^{-1} HCl, and concentrated Bradford dye reagent (BioRad Inc., Hercules, CA) was added. Sample absorbance was measured at 595 nm on a spectrophotometer at room temperature, and protein concentrations were calculated with a bovine serum albumin (BSA) standard curve.

Morphology

Morphometric analysis of echinoplutei total length from this experiment was presented previously (Yu *et al.,* 2011). Here, we supplement that data with additional morphological measurements. Samples were collected on day-3 (72 hpf) and day-6 (144 hpf) only, as these time points correspond to larvae having sufficient skeletal structure for measurements. Briefly, larvae were fixed in 1% sodium tetraborate ($Na_2B_4O_7$)-buffered formaldehyde in seawater and stored at 4 °C for no longer than 1 month after collection. Borate buffering maintains a high pH in the fixative and is commonly used in the preservation of calcium carbonate structures of marine zooplankton (Tucker and Chester, 1984). Larvae ($n = 30$) oriented with their dorsal side down against the slide were digitally photographed under bright-field DIC illumination at 10× on a compound microscope (Olympus BX50) with attached digital camera (Infinity Lite). Digital images were calibrated using a stage micrometer for the 10× objective in ImageJ (ver. 4.6). Total larval length was measured on both sides of each individual from the spicule tip of the postoral arm to the spicule tip of the aboral point (Yu *et al*., 2011). Postoral arm length was measured on the left side, from the spicule tip of the postoral arm rod to the intersection with the transverse body rod.

Data analyses

Data from this study are presented in five forms: (1) total lipid content and content of each lipid class; (2) ratios of TG to ST; (3) percentage of TG/ST remaining from the egg; (4) total protein content; and (5) larval morphometrics. In addition to absolute abundance of each lipid class, ratios TG/ST were used to quantify energy lipid content within each developmental stage. Sterol represents a structural lipid class that has been shown to remain stable during development in urchin larvae (Sewell, 2005; Yu, 2009), and is therefore suitable to help control for any variation in the number of individuals per sample. Since TG has previously been identified as an important energy storage lipid in this species (Meyer *et al.,* 2007; Adams *et al.*, 2011), our analyses quantified the amount of TG/ST present divided by the initial TG/ST present in the egg, resulting in the percent of TG/ST remaining compared to the endogenous supply (% TG/ST remaining). This metric served to quantify the proportion of endogenous energy resource remaining to fuel further development and is routinely used as an indicator of condition for larval fish and crustaceans (Fraser, 1989); thus this metric was utilized to provide normalization of TG content between females. Triglyceride is not known to be readily available for uptake from seawater, unlike amino acids, which can be absorbed by sea urchin embryos and larvae from the surrounding water (Manahan *et al.,* 1983; Shilling and Manahan, 1990; Meyer and Manahan, 2009). As such, total protein content does not represent a solely endogenous resource and is treated differently than TG in our analyses.

To investigate differences in lipid and protein in eggs, a one-way ANOVA was used with female as a fixed factor. To investigate differences in lipid and protein in embryos and larvae, a linear mixed model fit by maximum likelihood was used with "Stage" and "pCO_2" as fixed factors, blocked using "female" as a random effect, and incorporating "culture chamber" as a random effect. To investigate differences in postoral arm length at day-6, mean postoral arm length was calculated for each female and analyzed using a one-way ANOVA with "pCO_2" as a fixed effect. Although analyses of the data on total body length have previously been published (Yu *et al.,* 2011), they did not include measurements of postoral arm length. The analyses presented here expand on that previous work. Proportion data were arcsine transformed prior to analyses. Lipid (Total, TG, % TG/ST remaining, TG/ST) and postoral arm length data were power-transformed to meet the assumption of homogeneity of variance for ANOVA. Transformation of data to meet the assumptions for ANOVA was not possible for egg TG; these data were analyzed using a Kruskall-Wallis one-way analysis of variance by ranks test. When appropriate, *post hoc* analyses were performed using a Tukey Highly Significant Differences (HSD) test ($\alpha = 0.05$). Correlation analyses were conducted using Pearson's product-moment correlations. All statistical analyses were conducted using the R-Commander package, ver. 1.8 (Fox *et al.,* 2005) within R.

Results

Larval culturing and CO_2 parameters

Embryos of *Strongylocentrotus purpuratus* were successfully raised from single-cell embryos to the 4-arm pluteus stage in the experimental culture system. For each treatment, pCO_2 (μatm) was held relatively stable during the 6-day course of the culturing experiment (pCO_2: control, 365.4 ± 14.4; medium, 1038.9 ± 65.4; high, 1444.1 ± 52.0). On the basis of our calculated carbonate parameters, these values translated to pH of 8.07 ± 0.01, 7.67 ± 0.02, 7.53 ± 0.01, respectively (grand means based on data presented in Table 1). Under these conditions, estimated calcite saturation state remained saturated ($\Omega_{calcite} > 1$) for all treatments, while the high pCO_2 treatment (1450 μatm) was undersaturated with respect to aragonite ($\Omega_{aragonite} < 1$) (Table 1). Despite differences in carbonate chemistry, visual inspection of embryonic and larval stages confirmed that development proceeded normally and synchronously across treatments for all females (data not shown; see also Yu *et al.*, 2011).

Endogenous resources within eggs

Maternal energy provisions, in terms of lipid and protein content within unfertilized eggs, were quantified for each female (Fig. 1). Mean total lipid content in eggs from the four adult females was 15.67 ± 2.56 ng·ind^{-1} (mean ± s.d.). The majority of this total (59.2%) was composed of triglyceride (TG: 9.28 ± 1.23 ng·ind^{-1}), with phospholipid (PL: 31.1%, 4.87 ± 1.34 ng·ind^{-1}) and sterol (ST: 9.7%, 1.52 ± 0.29 ng·ind^{-1}) accounting for the remainder (Fig. 1). When normalized to ST, TG/ST was 6.25 ± 1.07 ind^{-1}. No difference was detected in total lipid, TG, TG/ST, or PL between females (ANOVA; total lipid: $F_{3,\ 8} = 1.75$, $P = 0.234$; TG/ST: $F_{3,\ 8} = 3.58$, $P = 0.066$; PL: $F_{3,\ 8} = 1.01$, $P = 0.437$; Kruskal-Wallis; TG: $H = 5.15$, $df = 3$, $P = 0.161$). Total protein content in eggs was 31.43 ± 3.29 ng·ind^{-1} (mean ± s.d.). A one-way ANOVA found significant differences in total protein content of eggs between females ($F_{3,\ 12} = 13.55$, $P = 0.0004$; Tukey: Female 1 < 4; Fig. 1).

Lipid analysis of early life-history stage

During early development, total lipid content decreased in embryos and larvae from all four females (Fig. 2). This decrease appeared to be primarily due to the depletion of TG, as levels of PL and ST remained relatively stable across all stages (Fig. 2). Although quantities of all lipid classes varied significantly across developmental stages, these levels were unaffected by pCO_2 (Table 2). *Post hoc* analyses revealed that both total lipid and TG differed significantly between all stages. Abundance of ST increased in day-3 (72 h) and day-6 (144 h) plutei compared to gastrula (Table 2).

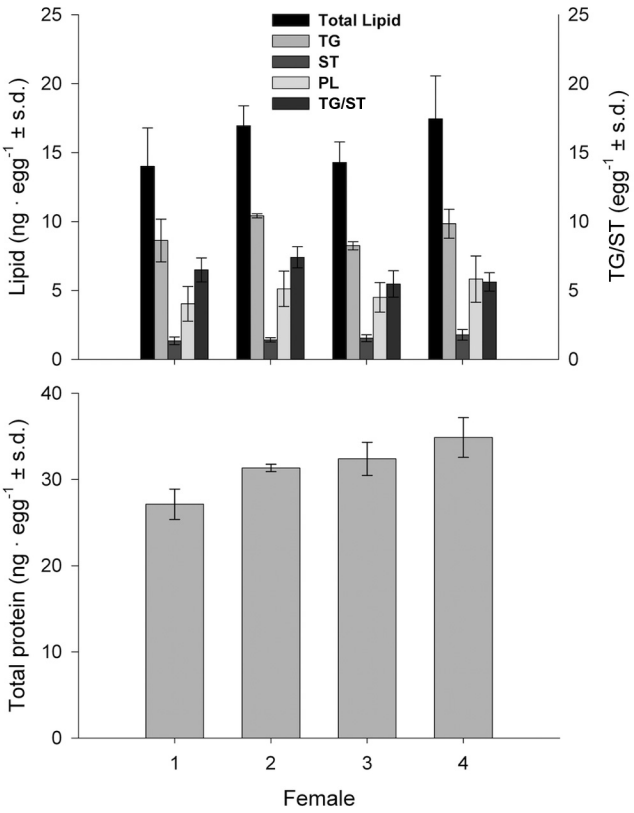

Figure 1. Variation in biochemical composition of eggs from different females in terms of (upper panel) total lipid, lipid components (TG = triglyceride, ST = sterol, PL = phospholipid), and the ratio of TG to ST, and (lower panel) total protein content. Data are presented as mean ng·egg^{-1} ± s.d. (lipid: $n = 3$; protein: $n = 4$).

A similar pattern was found regarding the percentage of TG/ST remaining, which was calculated by dividing TG/ST by the endogenous TG/ST present in the egg (see above). During development, the percentage of TG/ST remaining decreased at each sampling time-point in all four families, indicating the utilization of energy lipid reserves (Fig. 3). Across all treatments, embryos had 65% ± 19% (mean ± s.d.) of endogenous TG/ST remaining at the gastrula stage (25 hpf). This decreased to 25% ± 12% at the pluteus stage on day-3 (72 hpf) and 3% ± 2% on day-6 (144 hpf). While significant differences in the percentage of TG/ST remaining were detected between stages, no effect of pCO_2 was detected (Table 2).

Total protein content analysis

Although lipid levels decreased during development, total protein content remained fairly constant and was unaffected by pCO_2 (Fig. 4). Across all treatments, total protein content was 30.17 ± 3.21, 31.18 ± 2.67, and 29.92 ± 3.95 ng·ind^{-1} (mean ± s.d.) at the gastrula (25 hpf), day-3 (72 hpf), and day-6 (144 hpf) pluteus stages, respectively

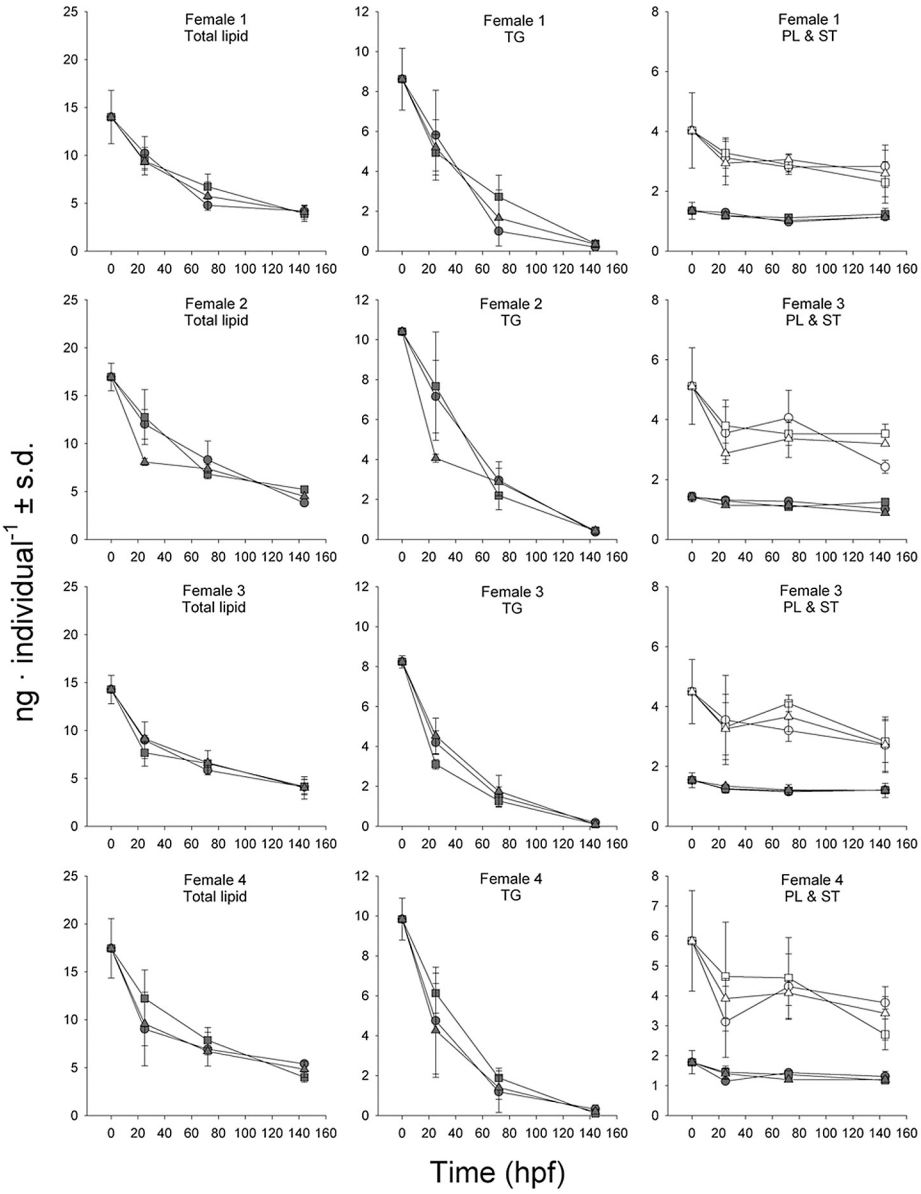

Figure 2. Variation in total lipid abundance in Females 1–4 during larval development at different pCO_2 conditions. Data are mean ng·individual^{-1} ± s.d. at each sampling point (n = 3, except for 365 μatm 25 hpf Female 4, 365 μatm 72 hpf Female 3, 365 μatm 144 hpf Female 3, and 1030 μatm 72 hpf Female 3, where n = 2; and 1450 μatm 144 hpf Female 2, where n =1). Different pCO_2 treatments are indicated by symbol: 365 μatm (circle); 1030 μatm (square); 1450 μatm (triangle). In the PL & ST panels, lipid classes are indicated by symbol color: phospholipid (white) and sterol (gray).

(Fig. 4). No differences were detected between developmental stages or pCO_2 levels (Table 2).

Larval morphometrics and correlations with egg composition

Analysis of morphological data found clear differences in postoral arm length at day-6 in response to pCO_2 (Fig. 5). Mean postoral arm length (± s.d.) was significantly reduced as pCO_2 increased (Fig. 5; 365 μatm: 187.0 ± 17.9 μm; 1030 μatm: 163.8 ± 18.3 μm; 1450 μatm: 154.3 ± 16.9 μm; ANOVA: $F_{2,\ 9}$ = 19.07, $P < 0.0006$; Tukey: 365 > 1030 = 1450). The relationship between postoral arm length and total length, expressed as the ratio of postoral arm length to total length, was altered by elevated pCO_2 (ANOVA: $F_{2,9}$ = 12.51, P = 0.003); *post hoc* analyses showed that larvae raised at 1450 μatm pCO_2 had signifi-

Table 2

Summary of results from multiple 3-way linear mixed model analyses fit by maximum likelihood of lipid and protein content in gastrula, day-3 pluteus, and day-6 pluteus stages

Variable	Factor	Df	χ^2	P ($>\chi^2$)	*Post hoc* ($P < 0.05$)
Total lipid	Intercept	1	1260.12	$\ll$ **0.001**	
	Stage	2	88.01	$\ll$ **0.001**	g ≠ d3 ≠ d6
	Treatment	2	3.13	0.209	
	Stage × Treatment	4	3.12	0.538	
TG	Intercept	1	75.14	$\ll$ **0.001**	
	Stage	2	146.74	$\ll$ **0.001**	g ≠ d3 ≠ d6
	Treatment	2	1.77	0.412	
	Stage × Treatment	4	5.43	0.246	
TG/ST	Intercept	1	53.64	$\ll$ **0.001**	
	Stage	2	150.02	$\ll$ **0.001**	g ≠ d3 ≠ d6
	Treatment	2	1.84	0.398	
	Stage × Treatment	4	6.50	0.165	
% TG/ST remaining	Intercept	1	4.34	**0.037**	
	Stage	2	123.00	$\ll$ **0.001**	g ≠ d3 ≠ d6
	Treatment	2	3.01	0.222	
	Stage × Treatment	4	6.46	0.167	
PL	Intercept	1	187.52	$\ll$ **0.001**	
	Stage	2	4.96	0.084	
	Treatment	2	2.45	0.294	
	Stage × Treatment	4	1.90	0.755	
ST	Intercept	1	789.53	$\ll$ **0.001**	
	Stage	2	7.71	**0.021**	g ≠ d3 = d6
	Treatment	2	0.51	0.775	
	Stage × Treatment	4	1.68	0.794	
Protein	Intercept	1	850.90	$\ll$ **0.001**	
	Stage	2	3.19	0.203	
	Treatment	2	0.16	0.922	
	Stage × Treatment	4	2.25	0.690	

Post hoc analyses (Tukey) were used to identify relationships between stages when significant differences within factors were detected (gastrula (25h), "g"; day-3 pluteus (72h), "d3"; day-6 pluteus (144h), "d6"). Relationships with $P \leq 0.05$ are indicated in **bold**.

cantly shorter arms relative to total length compared to control larvae at 365 μatm pCO_2 (0.473 ± 0.005 and 0.518 ± 0.043, respectively; Tukey: 365 μatm = 1030 μatm 1450 μatm).

To identify potential explanations for the observed morphological variation between pCO_2 treatments based on endogenous provisioning, we used linear regression to assess whether mean total larval length and mean postoral arm length could be predicted from mean endogenous levels of protein, total lipid, TG, and PL present in the eggs for each female. These analyses revealed a loss in the relationship (*i.e.,* decoupling) between mean egg PL content and total larval length at day-3 and day-6 under high pCO_2 (Table 3). Under control conditions (365 μatm pCO_2), mean egg PL explained a large and highly significant amount of variation in total length at day-3 (99.8%) and day-6 (97.3%). However, this relationship was not maintained at 1030 and 1450 μatm pCO_2 on day-3 or at 1450 μatm pCO_2 on day-6. Regression analyses suggest that postoral arm length at day-6 was not determined by egg lipids or protein in general, though a significant relationship was found with PL content at 1030 μatm pCO_2 (Table 3).

Discussion

This study examined the utilization of lipid and protein resources in fueling larval development in response to elevated pCO_2 in a temperate sea urchin, *Strongylocentrotus purpuratus*. The salient findings of this study were (1) lipid utilization rates and protein content in *S. purpuratus* did not vary across levels of pCO_2 ranging from 365 to 1450 μatm; (2) larval size was reduced at elevated pCO_2 despite similar rates of endogenous energy utilization; and (3) relationships between egg phospholipid content and larval length were found under control but not high pCO_2 conditions.

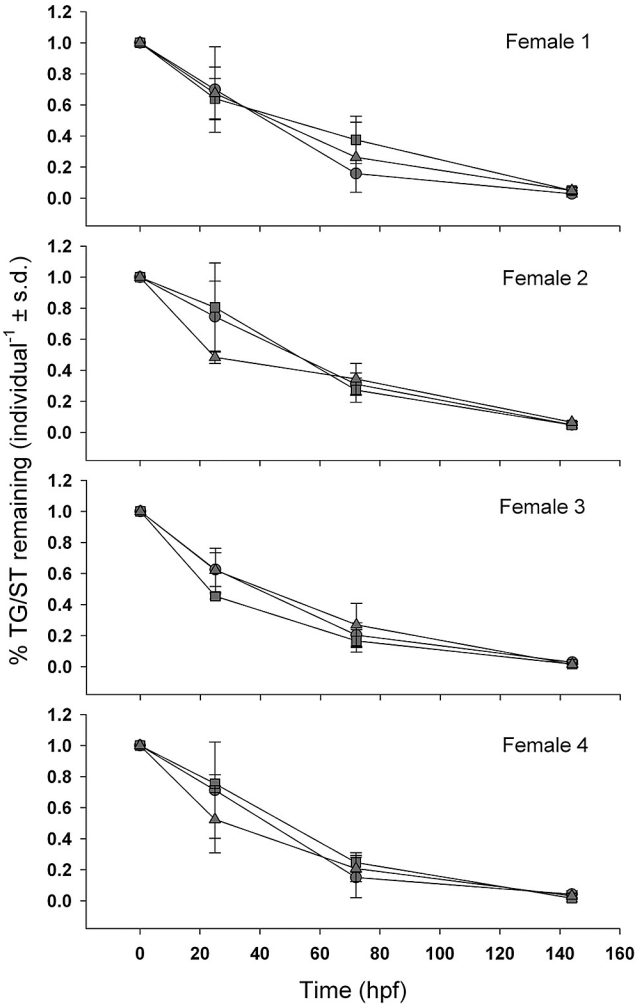

Figure 3. Patterns of lipid utilization from endogenous reserves during early development at different pCO_2 conditions for Females 1–4. Data are presented as mean proportion of triglyceride (TG) to sterol (ST) remaining from the egg ± s.d. ($n = 3$, except for 365 μatm 25 hpf Female 4, 365 μatm 72 hpf Female 3, 365 μatm 144 hpf Female 3 and 1030 μatm 72 hpf Female 3, where $n = 2$; and 1450 μatm 144 hpf Female 2, where $n = 1$) at each sampling point. Symbols indicate different pCO_2 treatments: 365 μatm (circle); 1030 μatm (square); 1450 μatm (triangle).

Lipid utilization and protein content in response to pCO_2

Using environmentally relevant values of pCO_2 (Yu *et al.,* 2011), we found that the biochemistry of purple sea urchin larvae was insensitive to elevated pCO_2 levels during early development. Despite a difference of $>$ 1000 μatm between the control and high pCO_2 treatments, we failed to detect any measurable changes in total lipid or total protein content between pCO_2 treatments during development. In addition, we failed to detect significant differences in utilization for any of the energetic (triglyceride [TG]) or structural (phospholipid [PL] and sterol [ST]) lipid classes between larvae raised under control conditions as compared to those raised under high pCO_2 conditions. The overall pattern of energy utilization during early development showed a rapid decline in total lipid content, primarily driven by a rapid decrease in TG with little to no change in PL and ST, and relatively stable protein content. This observation is similar to patterns of lipid utilization previously reported for *S. purpuratus* (Meyer *et al.,* 2007; Adams *et al*., 2011) and other temperate echinoids with planktotrophic larvae (Sewell, 2005; Byrne *et al.,* 2008; Yu, 2009). The absence of a pCO_2 effect on rates of energetic lipid or protein utilization may reflect a hard-wiring of energy utilization during early development that is robust to the environmental stressors used in this study. Additional experiments will be needed to sufficiently test this hypothesis.

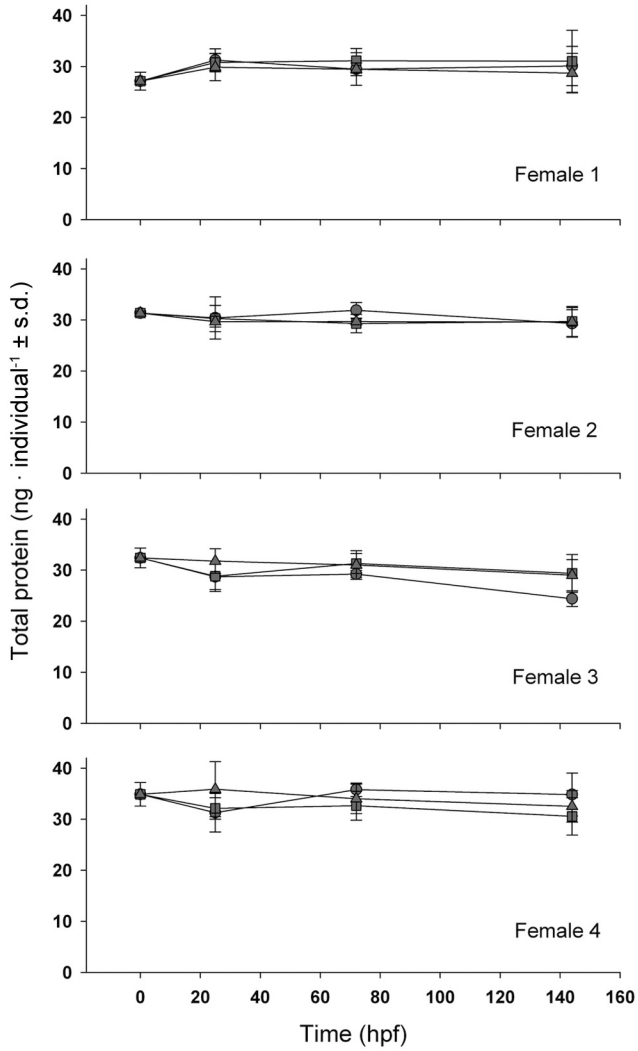

Figure 4. Patterns of total protein content during early development at different pCO_2 conditions for embryos/larvae from Females 1–4. Data are presented as mean total protein content ± s.d. ($n = 4$, except for 365 μatm 72 hpf Female 3 and 1030 μatm 72 hpf Female 2, where $n = 3$) at each sampling point. Symbols indicate different pCO_2 treatments: 365 μatm (circle); 1030 μatm (square); 1450 μatm (triangle).

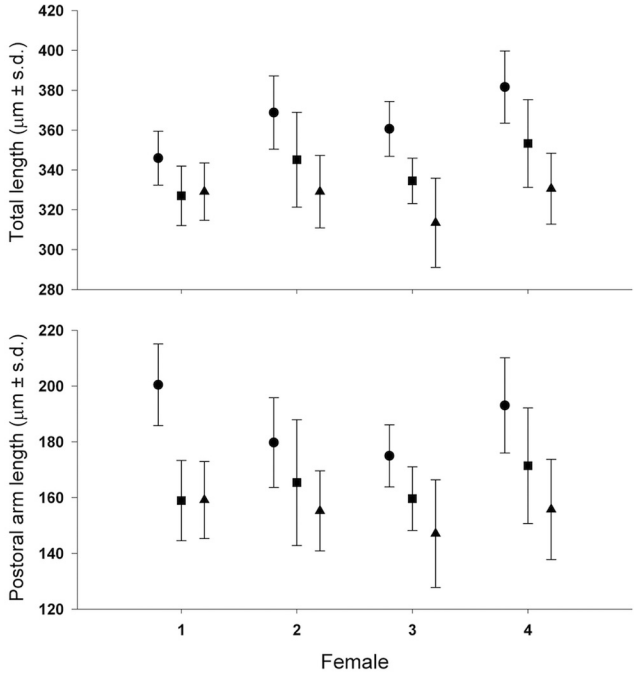

Figure 5. Morphological variation in day-6 (144 hpf) larvae exposed to different levels of pCO_2 in terms of total length (above) and postoral arm length (below). Data are presented as mean ± s.d. (n = 30) for each female. Symbols represent different pCO_2 treatments: 365 μatm (circle); 1030 μatm (square); 1450 μatm (triangle). Total length data are re-plotted from Yu *et al.* (2011).

Given its evolutionary history within this upwelling-dominated region where variable pH and relatively high pCO_2 is the norm (*e.g.,* Hales *et al.,* 2005; Feely *et al.,* 2008; Hofmann *et al*., 2011; Gruber *et al.,* 2012, Evans *et al.*, in press), *S. purpuratus* from this region may possess the physiological capacity to adequately resist exposure to moderately high pCO_2 water masses. Within the Santa Barbara Channel, where the adults for this study were collected, levels of pCO_2 have been shown to range between 250 and 850 μatm during summer (Yu *et al.,* 2011), with even higher values of pCO_2 (1100 μatm) during upwelling events along the California coast (Feely *et al.,* 2008; Hauri *et al.,* 2009; Fassbender *et al.,* 2011). These higher observed values exceed predictions of the Intergovernmental Panel on Climate Change of mean pCO_2 for the global ocean for 100 years in the future (Solomon *et al*., 2007), and this region is projected to experience severe undersaturation as early as 2050 (Gruber *et al*., 2012). Upwelling events occur sporadically during the season (spring-summer) in response to favorable winds that drive surface water offshore and generally persist on the scale of days (Pennington and Chavez, 2000). Additional ocean acidification (OA)-related investigations with other taxa, both from within and outside regions of upwelling, would be necessary to determine whether this response is conserved or is an environmental adaptation.

This study represents the first attempt to directly quantify the effects of OA on lipid utilization using biochemical-level methods commonly used in studies of larval physiology. Within the field of OA research, other studies have examined the role of lipid as a major energy source during development in marine invertebrates using more indirect measures. For example, using the C/N ratio as a proxy for total lipid, Walther *et al.* (2010) found a significant effect of CO_2 on crab megalopae, but only at very high CO_2 concentrations (3000 ppm *vs.* 380 ppm). Talmadge and Gobler (2010) used Nile Red staining to demonstrate a decrease in

Table 3

Summary of linear regressions between morphology (total length of day-3 and day-6 pluteus larvae and postoral arm length of day-6 larvae) and mean biochemical constituents in eggs from each female

		365 μatm		1030 μatm		1450 μatm	
Dependent variable	Biochemical Predictor	P	R^2	P	R^2	P	R^2
Day-3 (72h) Total length	Protein	0.153	0.718	0.667	0.110	0.745	0.065
	Total lipid	0.052	0.899	0.395	0.366	0.372	0.394
	TG	0.257	0.553	0.546	0.206	0.471	0.280
	PL	**0.001**	0.998	0.358	0.412	0.380	0.384
Day-6 (144h) Total length	Protein	0.071	0.864	0.145	0.732	0.875	0.016
	Total lipid	0.095	0.820	**0.038**	0.926	0.436	0.318
	TG	0.327	0.453	0.222	0.318	0.310	0.476
	PL	**0.014**	0.973	0.004	0.992	0.619	0.145
Day-6 (144h) PO length	Protein	0.580	0.176	0.238	0.581	0.540	0.211
	Total lipid	0.933	0.005	0.051	0.900	0.789	0.045
	TG	0.968	0.001	0.234	0.587	0.602	0.158
	PL	0.897	0.011	**0.021**	0.959	0.979	0.0004

Relationships with $P \leq 0.05$ are indicated in **bold**. TG, triglyceride; PL, phospholipid.

lipid index in pediveliger larvae of the clam, *Mercenaria mercenaria*, and the bay scallop, *Argopecten irradians*, as CO_2 concentrations increased from 250 to 1500 ppm; however, the authors attributed this change to reduced larval feeding efficiency. While these indirect methods provide useful proxies for total lipid content, they lack the ability to detect the composition and abundance of different lipid classes and distinguish between structural and energy storage lipids. This information may be important in unraveling how larvae respond to environmental stress from a biochemical perspective.

Larval size and relationship to biochemistry

While resource utilization remained similar across treatment levels, elevated pCO_2 did elicit a negative effect on larval size. As previously shown by Yu *et al.* (2011), the length of day-3 and day-6 plutei was significantly reduced under higher pCO_2 conditions. Similar OA-induced reductions in body size have been shown in other species with pluteus larvae (Kurihara *et al.*, 2004; Kurihara and Shiriyama, 2004; Dupont *et al.,* 2008; O'Donnell *et al.,* 2010; Yu, *et al*., in press). At day-6, the total length and postoral (PO) arm length of larvae cultured at 1450 μatm pCO_2 was reduced by 10% and 17%, respectively, compared to larvae cultured at 365 μatm. This differential reduction in length, as shown by a decrease in the ratio PO/total length, suggests that larval skeletons are not being reduced proportionately when developing under high pCO_2. Total larval length is composed of two separate skeletal elements, postoral arms and body rods. Increasing pCO_2 appears to have a greater effect on postoral arms than on body rods. Given the plasticity of arm length that has been previously shown in echinoid larvae in response to food concentration (Boidron-Metairon, 1988; Hart and Strathmann, 1994; McEdward and Herrera, 1999; Sewell *et al.,* 2004; Miner, 2007; Adams *et al*., 2011) and the associated costs of increasing length (Miner, 2007; Adams *et al.,* 2011), it may not be surprising that postoral arms may be preferentially reduced as the energetic costs to calcification become more challenging. Although nonlethal, an OA-induced reduction in size may have serious implications for swimming (Chan *et al.,* 2011; Clay and Grünbaum, 2011) and feeding performance (Hart and Strathmann, 1994; Hart, 1995), which may ultimately have consequences on metamorphic success and recruitment to benthic populations (Morgan, 1995).

Two possible mechanisms for the smaller sizes observed under elevated pCO_2 are reduced growth and developmental delay. Several studies have proposed that growth under OA conditions may incur a greater energetic cost (Cohen *et al.*, 2009; Melzner *et al.,* 2011; Stumpp *et al.,* 2011a). Organisms may have to either devote additional energy toward calcification at the expense of other processes, such as reduced muscle mass in adult brittlestars (Wood *et al.*, 2008), or reduce calcification rates as seen in larvae from a Mediterranean urchin (Martin *et al.,* 2011). Alternatively, developmental delay has been suggested as the mechanism for reduced larval size (Findlay *et al.,* 2009; Martin *et al.,* 2011; Stumpp *et al*., 2011b). Developmental delay can be difficult to identify without a stable reference against which to measure developmental progress. We are hesitant to rely on such a phenotypically plastic feature as postoral arm length as a marker for developmental timing. Instead, investigations of developmental delay would be better served using alternative methods, such as the timing of activation in developmental gene regulatory networks (*e.g.,* Davidson *et al.*, 2002; Hammond and Hofmann, 2012) or fine-scale developmental staging. We did not observe any noticeable delay in reaching major developmental markers (*e.g.,* blastula hatching or gastrulation) between pCO_2 treatments in this study, though these observations were not quantified. Previous work by Hammond (2010) did not find any delay in the deposition of amorphous calcium carbonate crystals during gastrulation in *S. purpuratus* in response to elevated pCO_2, and Padilla-Gamiño *et al.* (2012) found no evidence for developmental delay in growth of larvae raised under conditions of altered temperature and pCO_2 for this species.

In order to identify whether maternal provisioning influenced the observed morphological responses of larvae to elevated pCO_2, total and postoral arm lengths were compared to endogenous resources provisioned in the egg. It is interesting to note that eggs in this experiment have lower total lipid and higher total protein content compared to data presented by Meyer *et al.* (2007) for this species (lipid: 23.1 *vs.* 15.7 ng·ind^{-1}; protein: 31.4 *vs.* 12.9 ng·ind^{-1}). However, the TG content was comparable to that observed by Adams *et al*. (2011). This difference is likely due to differences in the adult food environment, which may affect the amount and quality of energy provisioned in the eggs. Notably, total lipid content of larvae at day-6 was similar to values reported by Meyer *et al.* (2007) of unfed larvae of the same age, approximately 5 ng·ind^{-1}. This congruence of results suggests a lower asymptote of total lipid content, primarily consisting of phospholipid, and differing percentages of total lipid used during development between the studies due to different starting amounts provisioned in the eggs. We observed a positive relationship between egg PL content and larval total length under control conditions (Table 3). This relationship ceased under high pCO_2 conditions, which we interpret as a potential stress response. However, care should be taken to avoid over-interpretation of this result; future investigations will be required to better understand the relationship between egg biochemical content and larval size.

Examining energy resources as they relate to growth and development is important because low pH may act as a stressor to some species and induce a metabolic cost for

general maintenance. Extrinsic environmental factors (such as pH) are known to cause energy to be allocated to areas other than growth, such as homeostasis. In marine calcifiers, increased pCO_2 causes a proton buildup within extracellular spaces, requiring increased energy to integral membrane ion pumps (*i.e.,* Na^+/K^+, Ca^{+2}, and H^+-ATPases) in order to maintain a favorable gradient for calcification (Pörtner *et al.*, 2005). This response may be even more exaggerated in osmoconforming invertebrate taxa, such as sea urchins, that have a low capacity for acid-base regulation (Pörtner, 2008; Spicer *et al.*, 2011; Stumpp *et al.*, 2012) particularly during ELS (Melzner *et al.,* 2009). The energetic demand from Na^+/K^+ ATPase activity alone has been estimated at 40% of the metabolic rate in pluteus-stage larvae of *S. purpuratus* (Leong and Manahan, 1997). To date, relatively little empirical work has investigated the effects of OA on larval metabolism (but see Albright and Langdon, 2011; Nakamura *et al.,* 2011; Stumpp *et al.,* 2011a). In general, results regarding effects of OA on metabolism have been mixed: responses appear to differ across taxa and life stages, with larval corals exhibiting metabolic depression (Albright and Langdon, 2011; Nakamura *et al.,* 2011) but juvenile and adult bivalves showing enhanced metabolic rates under elevated pCO_2 conditions (Beniash *et al.,* 2010; Cummings *et al.,* 2011). In *S. purpuratus,* evidence for metabolic depression has been shown in ELS (<5 days post fertilization; Padilla-Gamiño *et al.*, 2012) while older feeding larvae (>5 days post fertilization) have shown higher metabolic rates (Stumpp *et al.*, 2011a) in response to elevated pCO_2 conditions. If reduced larval size in response to increased pCO_2 was due to metabolic depression, then we expected to find more TG remaining in higher pCO_2 treatments. Conversely, if larval metabolism was elevated, as reported by Stumpp *et al*., (2011a), then we expected TG consumption to increase as pCO_2 increased. Instead, we observed that larvae developing under high pCO_2 had a smaller body size without a detectable increase in utilization of endogenous resources. We interpret our results as evidence that the larvae are either prioritizing energy directed toward development and physiological function at the expense of any increased costs associated with growth, or that reduced larval length is due strictly to higher costs of growth under OA. Additionally, recent studies comparing the natural variation between populations of *S. purpuratus* across its biogeographic distribution have shown that larvae have different responses to OA (Kelly *et al.*, 2012). Highlighting local adaptation, this outcome may explain why different results have been obtained for the same species; that is, the variable results for a single species may be resident in differences in physiology among and between spatially separated populations, such as those found either within or outside regions of intense upwelling.

Physiological responses may be further complicated by interactions between temperature and pCO_2 within the context of global climate change. Recent work has shown that increased temperature has a greater and sometimes opposite effect on metabolism than increased pCO_2 (Munday *et al.,* 2009; Lannig *et al.,* 2010; Sheppard Brennand *et al.,* 2010; Wood *et al.,* 2011; Stumpp *et al.,* 2011a; Nakamura *et al.*, 2011; Padilla-Gamiño *et al.*, 2012). While our biochemical data does not show any evidence of reduced energy utilization in larvae from high pCO_2 treatments compared to controls, future studies investigating metabolic demand in *S. purpuratus* using measurements of respiration and enzyme activity rates (*e.g.,* Padilla-Gamiño *et al.*, 2012) simultaneous with biochemical analyses are needed to identify OA-sensitive response mechanisms and understand the allocation of this endogenous energy supply within an OA-context. Developing embryos maintain a wide array of cellular defensive strategies to protect them from environmental variation, some of which are maternally derived and present within the egg (Hamdoun and Epel, 2007). Recent empirical evidence suggests that large geographical ranges alone may not ensure that species are protected from extinction due to changing climate conditions; adaptation potential may depend on the genetic variation within populations (Kelly *et al.,* 2011; Kelly and Hofmann, 2012). New tools are being developed that may help further examine gene-specific differentiation between populations at a genome-wide level (Pespeni *et al.*, 2010). The possibility exists that individuals or populations with the necessary phenotypic plasticity and genetic makeup to cope with OA may already be present in modern oceans (Sunday *et al.,* 2011; Foo *et al.,* 2012; Kelly *et al.,* 2012), and that these individuals may provide the foundation for future populations to persist under very different environmental conditions.

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