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Article

Environmental pH, O_2 and Capsular Effects on the Geochemical Composition of Statoliths of Embryonic Squid *Doryteuthis opalescens*

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Abstract: Spawning market squid lay embryo capsules on the seafloor of the continental shelf of the California Current System (CCS), where ocean acidification, deoxygenation and intensified upwelling lower the pH and [O_2]. Squid statolith geochemistry has been shown to reflect the squid's environment (e.g., seawater temperature and elemental concentration). We used real-world environmental levels of pH and [O_2] observed on squid-embryo beds to test in the laboratory whether or not squid statolith geochemistry reflects environmental pH and [O_2]. We asked whether pH and [O_2] levels might affect the incorporation of element ratios (B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, Pb:Ca, U:Ca) into squid embryonic statoliths as (1) individual elements and/or (2) multivariate elemental signatures, and consider future applications as proxies for pH and [O_2] exposure. Embryo exposure to high and low pH and [O_2] alone and together during development over four weeks only moderately affected elemental concentrations of the statoliths, and uranium was an

important element driving these differences. Uranium:Ca was eight-times higher in statoliths exposed to low pH_T (7.57–7.58) and low $[O_2]$ (79–82 $\mu mol \cdot kg^{-1}$) than those exposed to higher ambient pH_T (7.92–7.94) and $[O_2]$ (241–243 $\mu mol \cdot kg^{-1}$). In a separate experiment, exposure to low pH_T (7.55–7.56) or low $[O_2]$ (83–86 $\mu mol \cdot kg^{-1}$) yielded elevated U:Ca and Sr:Ca in the low $[O_2]$ treatment only. We found capsular effects on multiple elements in statoliths of all treatments. The multivariate elemental signatures of embryonic statoliths were distinct among capsules, but did not reflect environmental factors (pH and/or $[O_2]$). We show that statoliths of squid embryos developing inside capsules have the potential to reflect environmental pH and $[O_2]$, but that these "signals" are generated in concert with the physiological effects of the capsules and embryos themselves.

Keywords: market squid; statolith; geochemistry; deoxygenation; acidification; intensified upwelling; climate change; uranium

1. Introduction

Considerable environmental variation in upwelling ecosystems regularly exposes coastal fishery species to varying levels of pH, and $[O_2]$ through space and time [1–7]. Environmental fluctuations in seawater properties, including low levels of pH and $[O_2]$, can cause sublethal and lethal effects in loliginids (nearshore squid; [8]). Within squid habitats, low pH and $[O_2]$ seawater can be tightly associated with upwelling events [2]. Average pH and $[O_2]$ conditions can be further decreased in southern California during La Niña years as the thermocline shoals [1]. Early life stages of non-calcifying metazoans exposed to high levels of pCO_2 and associated low pH (e.g., acidification; [9–11]) are affected in several ways, including altered developmental, physiological and behavioral processes [12–15]. For cuttlefish and squid embryos, acidified environmental conditions generate additive effects, increasing an already acidified perivitelline fluid that baths embryos within the egg (cuttlefish) [16,17] and chorion (squid) [18,19]. Thus, environmental hypercapnia could even be more pronounced in early life stages. Further, some mollusks [20,21] and fish [22,23] are negatively affected when exposed to low levels of oxygen in their early life stages.

Environmental $[O_2]$ is decreasing more quickly along the coast of the Southern California Bight than in the offshore pelagic realm [24,25]. Ecological theory suggests that organisms will respond with species-specific shifts in size frequency and biogeographic range [8,26]. Many of the knowledge gaps regarding population level effects of $[O_2]$ and pH/pCO_2 exist because of inadequate tools for assessment, and geochemical proxies have not yet been utilized for squid.

The near-shore squid, *Doryteuthis opalescens*, is particularly sensitive to environmental change associated with the El Niño Southern Oscillation (ENSO; [27–29]), yet basic knowledge of the market squid population dynamics is lacking. This includes assessment of their population connectivity [30], as well as knowledge of critical ecological mechanisms controlling their population size [31]. Fishery boom and bust cycles often correlate with environmental change, such as that associated with ENSO [27–29]. Numerous hypotheses for annual catch fluctuation have been presented, but are difficult to test in the field because of sampling method biases [28,29,32]. The developmental,

physiological, behavioral and ecological mechanisms that lead to such drastic changes in annual catch have yet to be described, and the magnitude of risk for the population associated with environmental change is unknown. The embryonic life stage may be especially susceptible to low [O_2] and pH/pCO_2 exposure during La Niña events, because unlike other stages, embryos are site attached to the seafloor and are completely reliant upon a fixed energy reservoir (*i.e.*, yolk). Without the ability to move, embryos must tolerate exposure to low [O_2] and pH/pCO_2 levels using their fixed energy supply. One of the most promising tools available to investigators to understand environmental effects at the population level is through the use of statoliths [30,33,34] that are developed during this time.

Statoliths, made of ~95% $CaCO_3$ in the aragonite crystal form [35], are used by squid as part of their equilibrium and motion sensory organs (statocysts; [30,32,33,36]). Paired statoliths develop in each market squid embryo during the last two-thirds of the embryogenesis period (Figure 1; [37]), then remain embedded within the statocyst as it grows during each following life stage. After death, the statoliths can sometimes even be preserved in the fossil record [33,38,39]. Statolith aragonite crystal grows with a daily banding pattern, and growth is heavily influenced by the environment [33,34,38,40]. However, the squid statolith is not in direct contact with the environment, but rather with endolymph fluid within the statocyst [41].

Environmental chemical effects (e.g., [O_2] and pH) on embryos can be integrated with capsular effects. The structures of the capsule can connect the external chemical environment to the embryo via the capsular membranes, interstitial jelly and the chorion membrane (hereafter, these effects are collectively referred to as "capsular effects"). The pH/pCO_2 and [O_2] of the perivitelline fluid that surrounds the embryo are impacted additively by the environment and by the physiological processes of the embryo itself [17–19,42–47]. Elemental incorporation within statoliths can be influenced by the environment [30,33,34,48], but physiological process impacts on statolith geochemistry, including processes within the statocyst [41], embryo [18,19,45,46], as well as within outer-embryo structures [49,50], are not well understood. Further, the squid embryonic metabolic rate affects statolith formation [51,52]. The cephalopod-embryo metabolic rate greatly increases at the end of development [42,45,46] and is variable among embryos within the capsule [53]. The molluscan metabolic rate is highly influenced by temperature [45,46,54] and environmental oxygen [20,55,56]; cephalopods can be influenced by environmental pH/pCO_2 levels at the embryonic stages [45–47,52], but are tolerant at older stages [57,58]. As the statolith grows, the volume of the statolith increases exponentially. Thus, as a potential environmental recorder, the geochemistry of embryonic statoliths is weighted towards the end of benthic development. Glycoproteins, Sr^{2+}, Ca^{2+}, Mg^{2+} and HCO_3^- influence the biomineralization process of the squid [38]. Sr^{2+} is required for the initiation of statolith development [59], and Ca^{2+}, Mg^{2+} and glycoproteins are important for continued statolith growth [38].

Clear geochemical proxies for exposure to low pH have been established for foraminifera shells [60–62], coral skeletons [63] and mollusk shells [64] using either δ^{11}boron or uranium:calcium ratios. In addition, $\delta^{18}O$ has been explored as a proxy for O_2 in statoliths of *Illex illecebrosus* [35]. Here, we explore the element:calcium composition of market squid (*Doryteuthis opalescens*) statoliths as a potential proxy of pH/pCO_2 and [O_2] exposure using levels of pH/pCO_2 and [O_2] that reflect the highs and lows observed within embryo beds in southern California. This investigation is the first that we are aware of to test for a proxy of pH exposure using squid statoliths.

Any information about these squid could help to fill large knowledge gaps concerning scenarios of rapid climate change. Low pH/high pCO_2 and low [O_2] (hereafter referred to as "low pHOx") can cause species-specific negative effects in isolation or in tandem, and the magnitude of each of these effects is likely to be habitat specific [65,66]. We conducted experiments to investigate whether statolith geochemistry can reveal squid exposure to low pHOx, low [O_2] only and/or low pH/high pCO_2 only (hereafter, referred to as "low pH") during benthic encapsulated stages. We hypothesize that: (1) conditions associated with upwelled seawater observed in the *D. opalescens* spawning habitat influence the geochemical composition within embryonic carbonate structures in a manner useful as a proxy and (2) environmental exposure effects can be separated from capsular effects. More specifically, we assessed whether: (A) encapsulated embryos exposed to low pHOx yield distinct individual elemental levels in squid statoliths; (B) multi-elemental signatures can classify statolith exposures independent of individual elemental ratios; and (C) exposure to low pHOx levels yields elemental signatures different from individual effects of low [O_2] or low pH. A goal is to form the ability to assess the exposure of early developmental recruits collected from the field in the absence of seawater pH and [O_2] measurements.

2. Materials and Methods

Treatments were held constant over the entire embryogenesis period to allow for chronic exposure. Encapsulated squid embryos were reared under controlled conditions for the majority of embryogenesis. Experiment 1 (November, 2011) compared the statolith geochemical response between embryos exposed to high pH and [O_2] (high pHOx) and low pH and [O_2] (low pHOx) treatments, and Experiment 2 (March, 2012) compared the statolith geochemical response between embryos exposed to low [O_2] and high pH (low [O_2]) and low pH and high [O_2] (low pH) treatments. Levels were based on field measurements of water conditions made from water depths of 35–88 m water depth, ~6 km off of Del Mar, USA [1] (see System Overview and Experimental Treatments Section). Unlike near-surface waters, seawater in the lower to mid-shelf depths that are regularly utilized by squid [32] experiences reduced environmental variability (*i.e.*, environmental conditions are more stable). In the Southern California Bight, the magnitude of environmental variability (e.g., range of pH and [O_2]) decreases with depth. For example, the range of [O_2] decreased by 37% and the range of pH by 39% from a 7 to 17-m depth [3]. The variability [O_2] and pH continues to decrease with depth [1] near areas where squid-embryo capsules were collected (< 10 km). For embryo beds at 80–90 m on the shelf, pH and [O_2] conditions can be near constant over month-long periods. A novel laboratory approach using the Multiple Stressor Experimental Aquarium at Scripps (MSEAS; [67]) was used to control pH and [O_2] levels. Two experiments were conducted. Each included four tanks: two treatments with two replicate tanks each. For each experiment, capsules were randomized among treatments, aquaria and position within each aquarium.

2.1. Collection of Squid Embryos and Seawater Data

For Experiment 1, newly laid squid capsules (encapsulated embryos) were collected by hand from La Jolla Bay, San Diego, USA (32.86° N, 117.27° W), and from capsules laid at Scripps Institution of Oceanography (squid caught from Del Mar, USA; 32.96° N, 117.28° W). For Experiment 2, newly

laid capsules were taken from La Jolla Bay, USA (32.87° N, 117.25° W). All capsules were exposed to treatments for 24 days or more, allowing at least one week of exposure to treatment conditions prior to the initiation of statolith development (statoliths are completely grown under controlled conditions). Environmental data were collected continuously from a 30-m depth, 0.5 m above the seafloor, using a site-attached instrument (SeapHOx: described by Frieder *et al.* [3]) that measured conductivity, temperature, pressure, pH_T and $[O_2]$ from 23 June 2012 to 4 July 4 2013 (location: 32.86° N, 117.27° W).

2.2. System Overview and Experimental Treatments

Embryo capsules of *D. opalescens* in Experiment 1 were cultured under treatments of constant low (7.55, 90 μmol·kg^{-1}) and high levels (7.9, 240 μmol·kg^{-1}) of pH_T and dissolved oxygen (Table 1, Figure 1). Several step-wise changes in the system level setting were conducted during the course of each experiment to maintain stable environmental conditions [67], and thus, data were not normally distributed. Treatment conditions were distinct for each experiment with respect to pH, $\Omega_{aragonite}$ and $[O_2]$, but not for temperature and alkalinity (Wilcoxon test; Table 1). Capsules were cultured in 55-L aquaria that had been acid-rinsed in 1 N HCl and then rinsed five times with ultrapure H_2O (resistivity >18.0 MΩ·cm). Treatments were implemented using MSEAS, a manipulated flow-through aquarium design [67]. Seawater was supplied with seawater pumped from a 5 ± 1.5 m depth off of the Scripps Pier.

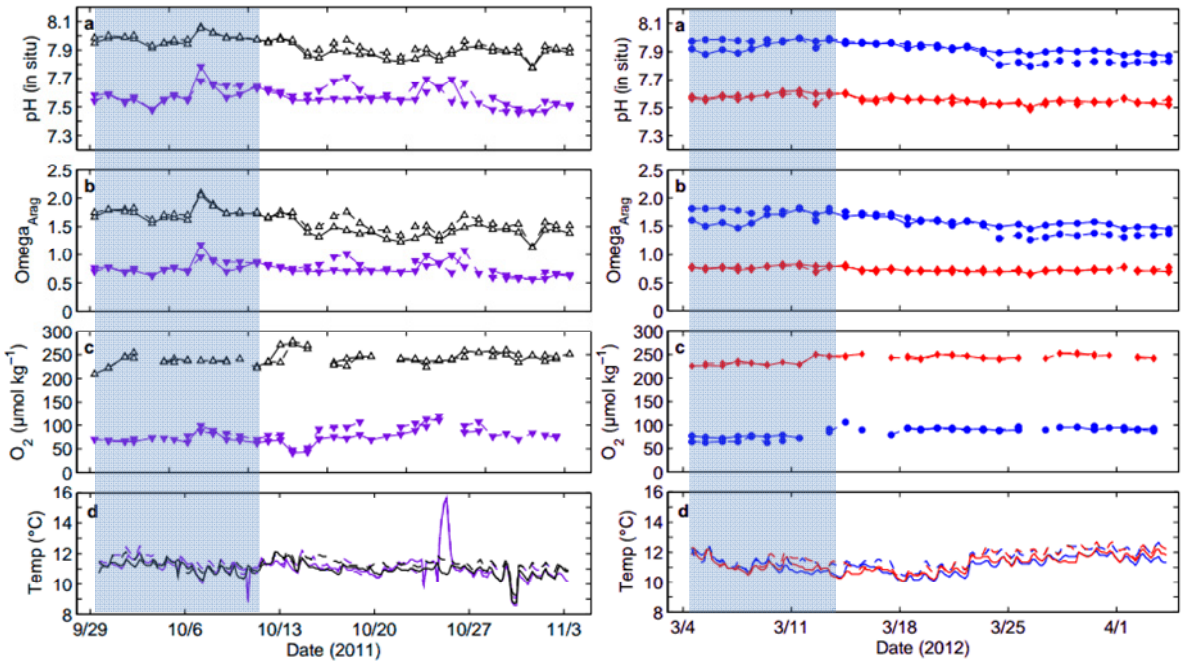
Table 1. Average ±1 standard deviation for temperature (°C), alkalinity (μmol·kg^{-1}), pH_T, and $[O_2]$ (μmol·kg^{-1}) in tanks during Experiment 1 and Experiment 2. Treatments were distinct for pH_T, $\Omega_{aragonite}$ and $[O_2]$, but not temperature and alkalinity (Wilcoxon test).

Treatment (Tank)	Temp (°C)	Alkalinity (μmol·kg^{-1})	$pH_{T\ (in\text{-}situ)}$ Total Scale	$\Omega_{Aragonite}$	$[O_2]$ (μmol·kg^{-1})
Experiment 1					
High pHOx (1)	11.3 ± 0.4	2215.5 ± 4.8	7.938 ± 0.053	1.62 ± 0.17	241.3 ± 12.3
High pHOx (2)	11.1 ± 0.4	2214.2 ± 6.6	7.916 ± 0.062	1.54 ± 0.21	242.6 ± 13.1
Low pHOx (1)	11.4 ± 0.8	2214.8 ± 6.3	7.578 ± 0.067	0.76 ± 0.12	82.1 ± 15.8
Low pHOx (2)	11.2 ± 0.9	2215.4 ± 5.8	7.567 ± 0.065	0.74 ± 0.12	78.6 ± 21.5
Treatment Effect (df = 1, N = 36)	$\chi2 = 0.02$, $p = 0.876$	$\chi2 = 0.01$, $p = 0.921$	$\chi2 = 109.35$ ***p* < 0.0001**	$\chi2 = 109.35$ ***p* < 0.0001**	$\chi2 = 90.76$, ***p* < 0.0001**
Experiment 2					
Low $[O_2]$ (1)	11.2 ± 0.5	2239.1 ± 5.5	7.923 ± 0.035	1.58 ± 0.10	86.4 ± 8.3
Low $[O_2]$ (2)	11.6 ± 0.5	2241.8 ± 4.5	7.908 ± 0.072	1.57 ± 0.21	83.0 ± 12.9
Low pH (1)	11.3 ± 0.5	2241.1 ± 5.8	7.559 ± 0.029	0.73 ± 0.04	241.1 ± 9.1
Low pH (2)	11.6 ± 0.6	2244.2 ± 7.1	7.552 ± 0.026	0.73 ± 0.04	241.7 ± 7.6
Treatment Effect (df = 1, N = 32)	$\chi2 = 0.05$, $p = 0.819$	$\chi2 = 3.14$, $p = 0.077$	$\chi2 = 93.74$, ***p* < 0.0001**	$\chi2 = 93.74$, ***p* < 0.0001**	$\chi2 = 72.74$, ***p* < 0.0001**

Embryos used for both experiments were collected from the field pre-organogenesis, Stages 11–12 [37,68,69]. For Experiment 1, 24 capsules were collected from the field just after being laid and were allowed to acclimate for 3 days at 11 °C. An additional 16 capsules laid in captivity at Scripps Institution of Oceanography were added to the experiment 7 days later. Ten capsules (6 field, 4 aquaria) were randomly assigned and placed into a position in each of four aquaria and were

evenly distributed among sources. Twenty capsules were placed into each aquarium. Densities in Experiments 1 (~100 capsules·m^{-2}) and 2 (density ~200 capsules·m^{-2}) are within the viable range found in the field, where they are reported to range from 1 to 47,720 capsules·m^{-2} [32,70].

Figure 1. Multiple Stressor Experimental Aquarium at Scripps (MSEAS) environmental data. From top to bottom, the graphs depict the (**a**) pH_T, (**b**) saturation state ($\Omega_{aragonite}$), (**c**) [O_2] (µmol·kg^{-1}) and (**d**) temperature (°C) of the seawater within each tank of (**A**) Experiment 1 and (**B**) Experiment 2. Purple = Low pHOx; Black = High pHOx; Blue = Low [O_2]; Red = Low pH. The graphic is modified from Bockmon *et al.* (2013) [67]. Blue shading = the estimated period prior to statocyst development (prior to the formation of the statolith).

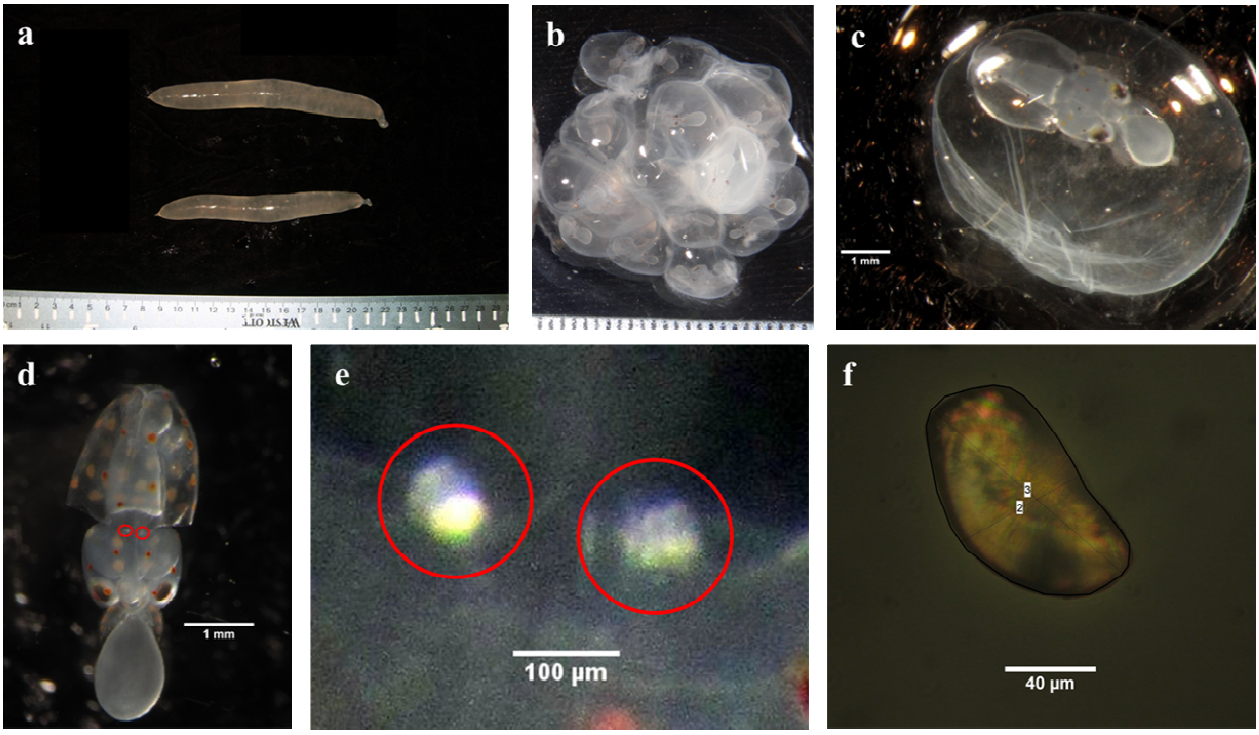


Embryos were cultured to embryonic-developmental Stages 28 or 29 (near-hatch paralarvae) [68] to reduce ontogenetic effects [71] and were collected only within the central portion of the capsule in order to reduce capsule-position effects [72] (Figure 2). These stages were indicated by the pigmentation of the ink sac (this occurs earlier in *D. opalescens* than in *D. pealeii*), the complete covering of the eyes by the cornea, but not a prominent Hoyle's Organ [37,68,69]. Cultures were maintained at constant temperature (11.3 °C ± 0.3 °C, SD), salinity (33.4 ± 0.2) and light levels using 15 W LED lights on a 12:12 h light:dark cycle to reduce these types of environmental effects on statolith development [21,40,72]. Salinity, temperature and seawater flow rate were constant among treatments. Statolith development takes place at the start of organogenesis [37], and our experiments exposed embryos to treatments one week or more prior to the statolith formation. The low [O_2] and pH treatments developed more slowly (5–7 d) compared to the other treatments; thus, samples from the 3 treatments (Experiment 1: low pHOx and Experiment 2: low pH, low [O_2]) were gathered at two times, once to match the high pHOx treatment exposure duration and once again 5–7 d later to allow embryos

exposed to low [O_2] and/or pH to develop to near-hatch Stages 28–29. The levels of [O_2], pH, $\Omega_{Aragonite}$ and temperature were held constant for each treatment (Figure 1).

Further, to verify whether or not seawater trace-metal concentration varied among treatments, 100-mL seawater samples were taken weekly using clean-lab protocols to minimize any possibility of contamination [73]. Seawater samples were filtered, acidified with 50 µL of 12 N optima HCl and stored in darkness at room temperature (21 °C ± 3 °C) until they were analyzed at Arizona State University for magnesium^{24}(Mg), calcium^{48}(Ca), strontium^{88}(Sr), barium^{138}(Ba) and uranium^{28}(U). Boron (B) was estimated using discrete salinity values taken daily [74].

Figure 2. (**a**) Two squid-embryo capsules. Capsules are directly exposed to the environment, and each contains between 100 and 300 embryos. Ruler units are in cm; (**b**) Subsection of the capsule with the capsular membranes removed. Gelatinous material fills the interstitial space between the chorions and the capsular membranes; tick marks at the bottom of the image demarcate mm; (**c**) Chorion filled with perivitelline fluid and containing a squid embryo; (**d**) Squid embryo: statocysts are circled in red; (**e**) Statocysts are filled with endolymph fluid, and each contains a single statolith; (**f**) Embryonic statolith, made of aragonite.



2.3. Extraction and Mounting of Statoliths for Elemental Analyses

Statolith extraction and mounting procedures followed clean lab protocols [73]. Statoliths were removed for analysis when embryos were developed to Stages 28–29 [68]. Statoliths were removed by dissection, and then chemical digestion of soft parts (encapsulation and embryo proteins) was carried out by placing embryos on a slide with digesting solution (10 µL of ultrapure water and 5 mL of ultrapure 15% H_2O_2 buffered with 0.05 N NaOH in ultrapure H_2O) for 10–20 min, depending on the amount of soft tissue. Digestion solution was removed by pipetting with a clean tip, and statoliths were

rinsed three times in ultrapure H_2O. All remaining H_2O was removed by evaporation overnight underneath a hood within a Class 100 clean room. One statolith from each embryo was extracted, and 10–20 embryos were dissected from the center position of each capsule (total = 10–20 statoliths from each capsule). Statoliths were mounted on double-sided tape (Scotch^{TM}), attached to a slide and prepared for laser ablation. The chemical composition of mounting tape was determined by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) [75]; all elemental counts from the tape were at least three orders of magnitude lower than counts found in statoliths. Statolith lengths ranged from 65 to 130 μm.

2.4. LA-ICP-MS Instrument Settings and Methods for Elemental Analyses of Statoliths

Statoliths were analyzed by LA-ICP-MS at low resolution for an elemental menu consisting of boron^{11} (B), magnesium^{24} (Mg), calcium^{48} (Ca), manganese^{55} (Mn), copper^{63} (Cu), zinc^{66} (Zn), strontium^{88} (Sr), barium^{138} (Ba), lead^{208} (Pb) and uranium^{238} (U). Ablated material from haphazardly selected statoliths from each slide was introduced via a New Wave UP-213 UV laser, frequency-quadrupled to a 213-nm wavelength with a nominal beam width of 40 μm in the spot beam setting, into a Finnigan Element 2 sector field ICP-MS using a micro-flow nebulizer at 20 $\mu L \cdot min^{-1}$. The laser was set to the spot beam mode at 40% power and a frequency of 20 Hz. Consistent plasma conditions were maintained using 1% HNO_3 during analysis of standards, on instrument blanks and on laser-ablated samples [75]. Instrument sensitivity was monitored measuring indium (In) and was approximately 1×10^6 counts$\cdot s^{-1}$ for 1 ppb. All slides were assigned a number and then analyzed in a random order. The laser-ablation process completely vaporized each statolith.

To standardize the mass variation of statolith samples, the concentrations of elements in the statoliths are reported as ratios with respect to Ca, the dominant elemental constituent of the statolith [76]. Aragonite is a solid, acellular, metabolically inert, crystalline structure [76] that is ~95% $CaCO_3$ [35] and differs from other calcified structures found in cephalopods that are porous, cellular and metabolically active, such as cuttlebone, which have been shown to become less porous (hypercalcification or increased $CaCO_3$ density) in response to hypercapnia [47,77]. Further, if hypercalcification or hypocalcification is induced by a treatment, we would expect all element:calcium ratios to be significantly increased (hypocalcification) or decreased (hypercalcification). Therefore, we would predict that this method is sensitive to changes in calcium concentration. Element:Ca of the sample was determined by using matrix-matched solution standards of known element:Ca and a mass-bias correction [78].

Several steps were taken to ensure accurate element:Ca of the samples (*i.e.*, the error in measurements using the ICP-MS and laser ablation unit). Throughout the analyses, the ICP-MS tested solution-based measurements, which remained within 5% of each element's known values. Using a solution standard containing Ca, Mg, Sr, Ba, Ce, Pb, U, Mn and Zn (Spex Certified primary standard solutions), values of the ICP-MS measurements compared to known values were as follows: Mg:Ca (mmol:mol) = 1.24%, Sr:Ca (mmol:mol) = 0.37%, Ba:Ca (μmol:mol) = 2.77%, Pb:Ca (μmol:mol) = 3.09%, U:Ca (μmol:mol) = 0.40%, (N = 20)). The accuracy of the laser ablation method was estimated by using glass standard number 612 from the National Institute of Standards and Technology, Gaithersburg, USA (NIST612). The repeatability (relative standard deviation, % rsd) of

the method was determined using the results of the laser-ablated NIST612 standard reference material, except for Sr:Ca, which was determined using the otolith material: B:Ca (mmol:mol) = 0.02%, Sr:Ca (mmol:mol) = 0.61%, Ba:Ca (μmol:mol) = 0.18%, Pb:Ca (μmol:mol) = 11.13%, U:Ca (μmol·mol^{-1}mol) = 4.60% (N = 12). Accuracy for B:Ca was calculated using NIST612, because carbonate and solution-based standards were unavailable, leaving the potential for matrix effects.

Detection limits were defined as the intensities (counts per second) of elements present in the instrument blank plus three times the standard deviation [79]. The average intensity of each element above the detection limit was as follows (measured as multiples of detection limit): Mg > 1770, Sr > 1,280, Ba > 440, U > 220, Pb and Cu > 50, Zn > 40, Mn > 1.2 and B > 0.8. The percentage of samples where each element was above the detection limit was as follows: Mg 97%, Sr 99%, Ba 100%, U 97%, Pb 98% and B 40%. For the B data, we only included those samples that were above detection limits and eliminated all others from further analysis.

2.5. Statistical Analyses of Seawater and Statolith Elemental Composition

Seawater samples were taken weekly from each tank within each treatment. For statolith samples, tanks were considered replicates for statistical testing of the hypotheses. The average number of embryos (*i.e.*, statoliths) analyzed per capsule was 5.4 ± 0.5 (standard error). All data were first examined for variance homogeneity and tested for normality by means of residual analysis prior to using ANOVA models. All seawater elemental data were normal, and statolith elemental data were normal for B:Ca, Sr:Ca and Ba:Ca (no transformations needed). Statolith Mg:Ca, Pb:Ca and U:Ca data were right-skewed, with U:Ca being the most intensely skewed of the data sets. Mg:Ca and Pb:Ca data were square-root transformed, whereas U:Ca data were cube-root transformed; data were normally distributed after transformation. Experiments 1 and 2 were tested separately. Treatment effects were tested using a one-way mixed model ANOVAs nesting the tank (fixed) and capsular (random) factors [80]. In Experiment 1, the effects of treatment on the elemental concentration (element:Ca) of statoliths were tested between groups with high pHOx to those of low pHOx. In Experiment 2, the effects for treatment onto the elemental concentration (element:Ca) of statoliths were tested between groups with low [O_2] and low pH levels.

Multivariate analysis of similarity (ANOSIM, Euclidean Distance, N = 9999 permutations) was used to test the hypothesis that the treatment influences the multi-elemental composition of the statoliths. Principle component analysis (PCA) was used to investigate elemental signatures among groups using a correlation matrix (*i.e.*, data were not transformed). Statistical analyses were conducted using JMP (Version Pro 11) statistical software (SAS Institute, Cary, NC, USA).

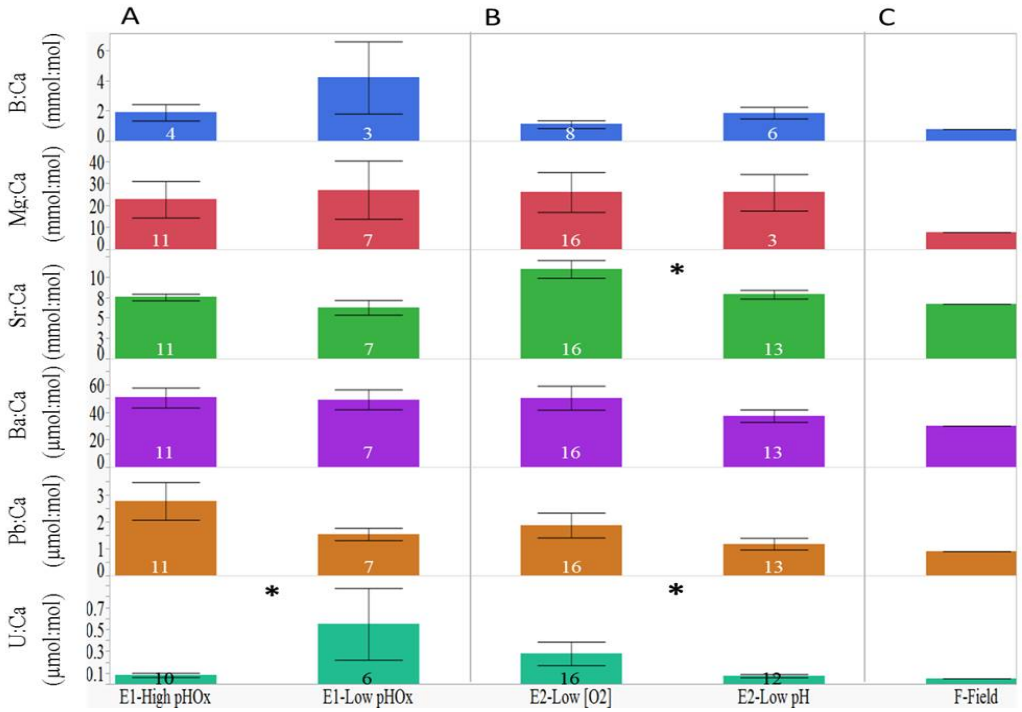
3. Results and Discussion

Seawater element concentrations were not different among treatments in Experiment 1 or Experiment 2 (Appendix Table A1). Further, chronic exposure to undersaturated seawater ($\Omega_{aragonite} < 1$; Table 1) did not prevent statolith formation. Six elements measured within the statoliths had concentrations detectable at levels sufficient for analyses: B, Mg, Sr, Ba, Pb and U. This is the first report of B being detectable within statoliths of the cephalopod taxon and the first time U has been reported as detectable within statoliths of *D. opalescens*. Further, several patterns emerged through elemental analyses.

3.1. Elemental Variations among Treatment Groups

In Experiment 1, U:Ca was significantly higher (eight times) within the statoliths from the low pHOx treatment ($F_{1,3}$ = 16.86, p = *0.0005*) than the high pHOx treatment (Figure 3). The only tank effect observed was also for U:Ca in Experiment 1 ($F_{2,3}$ = 5.42, p = *0.0130*), but this was driven by capsular effects ($F_{3,13}$ = 2.65, p = *0.0060*) and not by seawater effects ($F_{1,4}$ = 0.0302, p = 0.86; Appendix Table A1). Statolith U:Ca averages among capsules in the low pHOx treatment varied over an order of magnitude (capsule values = 0.357, 0.343, 0.248, 0.157, 0.021 µmol/mol). No other individual elements significantly varied between treatments in Experiment 1, and we did not collect any evidence to support a change in calcium concentration indicative of either hypercalcification [47,77] or hypocalcification. Experiment 2 was conducted to reveal whether or not low pH only or low [O_2] treatments induced a similar or distinct response. In this experiment, the low [O_2] only treatment elicited a distinct response of U:Ca relative to embryos exposed to low pH only ($F_{1,6}$ = 5.91, p = 0.0225; Figure 3). The endolymph fluid within the statocyst is highly regulated in squid [41] with similarities to the highly-regulated sacculus found in fish [81,82]. Low environmental [O_2] may impair the regulation of internal pH by embryonic squid, due to the reduced aerobic metabolic rate. This can lead to insufficient ATP production necessary to fuel active mechanisms for pH regulation and calcification.

Figure 3. Element:Ca for each treatment analyzed. (**A**) Experiment 1: Low pHOx results in statoliths with high levels of U:Ca ($F_{1,\ 3}$ = 16.86, p = *0.0005*); (**B**) Experiment 2: Low [O_2] results in a higher U:Ca concentration within statoliths in comparison to low pH ($F_{1,\ 6}$ = 5.91, p = *0.0225*) and higher Sr:Ca concentrations ($F_{1,\ 6}$ = 6.47, p = 0.0174); (**C**) Field values are from a single capsule that developed in the field. Treatments were tested using one-way ANOVA. Number within column = Number of capsules analyzed. * Significant. Bar = ±1 standard error from the mean.



Interestingly, statoliths from the low-[O_2] treatment had a higher Sr:Ca ratio in comparison to the low-pH treatment ($F_{1,6}$ = 6.136, p = *0.021*; Figure 3). Environmental strontium is critical for statolith formation [59]. Strontium has been associated with temperature effects and salinity effects, but this is the first report of a strontium effect associated with [O_2] or pH. No other elements exhibited treatment effects.

The capsular effects (integrated effects of outer-embryo structures, capsular and chorion membranes and embryonic processes) were significant for all element:calcium ratios for each experiment (Table 2).

Table 2. The statolith elemental:calcium composition among capsules within treatments was tested using a one-way mixed model ANOVA (nested within treatment and tank factors). Significance = bold.

Element:Ca	Experiment 1	Experiment 2
B:Ca	$F_{3, 4}$ = 6.14, $\mathbf{p < 0.0001}$	$F_{6, 10}$ = 17.43, $\mathbf{p < 0.0001}$
Mg:Ca	$F_{3, 14}$ = 20.15, $\mathbf{p < 0.0001}$	$F_{6, 25}$ = 45.07, $\mathbf{p < 0.0001}$
Sr:Ca	$F_{3, 14}$ = 13.27, $\mathbf{p < 0.0001}$	$F_{6, 25}$ = 41.74, $\mathbf{p < 0.0001}$
Ba:Ca	$F_{3, 14}$ = 2.82, $\mathbf{p = 0.0026}$	$F_{6, 25}$ = 51.12, $\mathbf{p < 0.0001}$
Pb:Ca	$F_{3, 14}$ = 31.05, $\mathbf{p < 0.0001}$	$F_{6, 25}$ = 39.92, $\mathbf{p < 0.0001}$
U:Ca	$F_{3, 13}$ = 2.65, $\mathbf{p = 0.0060}$	$F_{6, 25}$ = 103.28, $\mathbf{p < 0.0001}$

The capsular effects are significant for several reasons. First, these findings support the importance of the direct and/or indirect maternal influence on embryonic statolith geochemistry. Evidence for the direct maternal transfer of two essential elements (^{75}Se, ^{65}Zn) and one non-essential element ($^{110\,m}$Ag) has been reported for a cuttlefish [83]. Other studies have found evidence indicating a direct role of maternal transfer to embryonic statoliths [73,84]. Indirect maternal influence could be caused by a variation in the quality of the capsular and chorion membrane. Although this issue has not been explicitly studied, other investigators have found significant differences of Co uptake among capsules (one capsule of three capsules) of the squid, *Loligo vulgaris* [49]. Second, our findings show that the embryonic-statolith geochemistry is distinct for many elements among capsules, but not distinct for most elements among environmental treatments. These differences may be attributable to differences among capsular units. Each unit may have differences in: (1) elemental uptake in their capsular or chorion membranes [49,50]; (2) utilization of embryonic-epidermal ionocytes [17–19]; (3) embryonic metabolism [45,46] and the effect total embryo metabolism per capsule has on the diffusion of environmental [O_2] and pCO_2 [20,55,56]; and (4) the rate of active transport of the statocyst membrane [41]; or (5) any combination these factors. Although there are many reports that support environmental "recording" within statolith geochemistry [30,33,34,59], our data suggest that statolith geochemistry records both the environment and capsular effects within each embryo.

3.2. Multivariate Analyses

To test whether the elemental composition of statoliths varied among treatments, multivariate analyses were conducted for each experiment using analysis of similarities (ANOSIM; one-way, Euclidean-distance matrix, N = 9999 permutations) and principle component analysis (PCA). The

elemental composition between treatments was not distinguished in either Experiments 1 or 2 (ANOSIM: Experiment 1, R = 0.022, $p = 0.369$; Experiment 2, R = 0.007, $p = 0.359$).

These multivariate results presented emphasize a limited relationship between statolith elemental chemistry and the environmental pH and [O_2]. ANOSIM analyses were used to compare statolith chemistry among capsules within treatment (total of four tests; Table 3). Again, the capsules exhibited a significant effect on statolith chemistry within each treatment group, with the exception of the low pHOx treatment (although no effect was detected, this may be caused by a lack of statistical power associated with a small sample size). Elements driving capsular differences were not similar between the experiments (Table 4, Figure 4). These data indicate that any statolith-geochemical record would be an integrated signal between the environmental pH and [O_2] and physiological processes within outer embryonic structures.

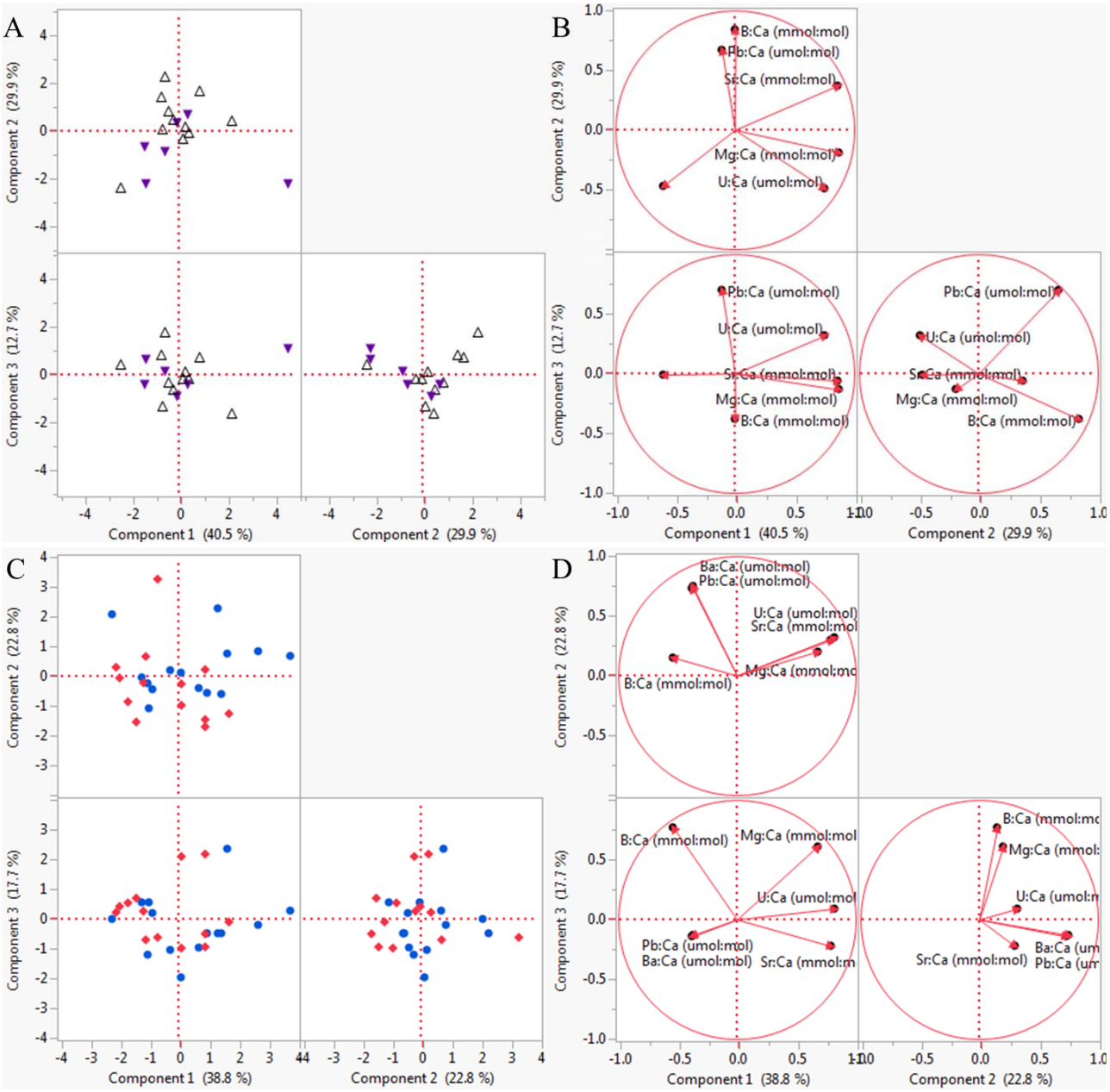
Table 3. Statolith elemental composition differences among capsules within each treatment were tested using a one-way analysis of similarities (Euclidean-distance matrix; elemental menu = B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, U:Ca; N = 9999 permutations for all treatments, except low pHOx (N = 3)).

Groups	R Statistic	*p*-Value
High pHOx	0.890	***0.010***
Low pHOx	1.000	0.333
Low [O_2]	0.920	***0.010***
Low pH	0.892	***0.010***

Table 4. Elements driving compositional differences among treatments (correlation matrix; elemental menu = B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, Pb:Ca, U:Ca). Eigenvalues and the percentage of variance explained are listed beneath each principle component (PC). Bold lettering = significant.

Experiment 1	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Element	**(2.43, 40.5%)**	**(1.79, 30.0%)**	**(0.76, 12.7%)**	**(0.64, 10.7%)**	**(0.20, 3.3%)**
B:Ca (mmol:mol)	0.00280	**0.62922**	**-0.43943**	0.38658	**0.47883**
Mg:Ca (mmol:mol)	**0.55641**	−0.14115	0.51101	**0.40241**	**−0.47422**
Sr:Ca (mmol:mol)	**0.55046**	0.27674	0.18102	0.05333	−0.18725
Ba:Ca (µmol:mol)	−0.38689	−0.35043	0.37102	**0.78068**	−0.04598
Pb:Ca (µmol:mol)	**−0.06976**	**0.50151**	**0.80384**	0.24169	−0.15971
U:Ca (µmol:mol)	**0.48254**	−0.36505	0.36425	0.13378	**0.69485**
Experiment 2	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Element	**(2.33, 38.8%)**	**(1.37, 22.7%)**	**(1.06, 17.7%)**	**(0.57, 9.6%)**	**(0.37, 6.2%)**
B:Ca (mmol:mol)	−0.35216	0.13043	**0.74688**	0.08813	0.21714
Mg:Ca (mmol:mol)	**0.44385**	0.17315	**0.59349**	−0.07149	0.06847
Sr:Ca (mmol:mol)	**0.52043**	0.25779	−0.21675	0.15301	**0.71352**
Ba:Ca (µmol:mol)	−0.24548	**0.64466**	−0.12698	**0.66566**	−0.15275
Pb:Ca (µmol:mol)	−0.24588	**0.62852**	−0.13803	**−0.71931**	0.08053
U:Ca (µmol:mol)	**0.53611**	0.27552	0.08822	−0.05656	**−0.63972**

Figure 4. Principal component analyses. Experiment 1: (**A**) Score plots among treatment groups; (**B**) Loading plot (correlation matrix, elemental menu = B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, U:Ca). Purple = low pHOx; Black = high pHOx. Experiment 2: (**C**) Score plots among treatment groups; (**D**) Loading plot (correlation matrix, elemental menu = B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, U:Ca). Blue = low [O_2]; Red = low pH.



3.3. Statoliths as an Indicator of Environmental Response

The experiments conducted here provide the first evidence that embryonic statolith geochemistry can be affected by environmental [O_2] and pH at levels that occur at natal sites. B:Ca and U:Ca were investigated as pH proxies; however, they did not exhibit the direct relationship with environmental pH that has been found with foraminifera shells [60–62], coral skeletons [63] and mollusk shells [64]. These B:Ca results suggest that, when environmental pH_T is low (7.55–7.56) and [O_2] is high

(241–242 μmol·kg^{-1}), squid embryos can regulate the endolymph pH within the statocyst, where the statolith crystal grows. Constant pH was found in a study of the endolymph fluid of squid statocysts [41], and pH has been shown to be highly regulated in the endolymph fluid of saccules of fish (the squid statocyst analog) [81,82]. Lower taxonomical groups have a more direct relationship with seawater, with less integration of physiological processes [76]. Seawater with low pH_T has higher levels of bioavailable U, and in foraminifera, the incorporation of U into biogenic $CaCO_3$ increases with decreasing [CO_3^{2-}] ([61,62,85]). Because we did not find evidence of U enrichment within statoliths in the low-pH treatment, we presume that [CO_3^{2-}] is also regulated within endolymph fluid.

These results suggest that both pH and [CO_3^{2-}] are highly regulated within the squid embryos and the statocyst, as has been found with other squid [41], and these regulation processes are unaffected by an environmental pH_T level of 7.55. One option is that high pCO_2/low pH in seawater does not significantly affect the blood chemistry of squid embryos [86], due to ion-regulating epithelia regulating internal pH [17–19] (Figure 2). However, it is also possible that squid embryos exposed to high pCO_2/low pH can utilize energy derived from yolk reserves to compensate for putative alterations in their internal pH. Further testing is needed to determine if there is a threshold below which squid are not able to regulate their pH. Moreover, it is essential to know if the *D. opalescens* embryo is internally acidified during exposure to realistic, high pCO_2/low pH conditions and the compensatory mechanisms that are involved.

Development of an environmental [O_2] proxy is still in its infancy, and more research is needed to test for different mechanisms. However, U:Ca and Sr:Ca were enriched in squid statoliths grown in low [O_2] treatments (Figure 3). Environmental strontium is critical in the formation of the statolith [59]. Sr:Ca and Ba:Ca are widely reported to have a strong, often negative, relationship with temperature, although for Sr:Ca, the relationship can be more complex [87]. U:Ca was recently reported to have a positive relationship with temperature [88]. Since all tanks were kept within 1 °C of one another (Figure 1) and did not reach the temperature differentials reported to generate Sr signals for a congener squid species (>2 °C, [48]) and one gastropod (= 4 °C, [73]), our results are not likely related to temperature effects. Curiously, when exposed to low [O_2] with low pH (low pHOx), statoliths were not enriched with Sr. Strontium incorporation into squid statoliths may be inversely related to metabolic rate. Low environmental pH/high pCO_2 and high temperatures can cause metabolic depression in squid embryos [45,46] and reduced growth [52].

The only element:calcium measured in this study that might be a useful indicator of low pHOx conditions is U:Ca. We showed that U:Ca is enriched (eight-fold increase) in the statoliths of embryos exposed to low pHOx relative to those exposed to the high pHOx treatment. We propose that this enrichment is driven by low [O_2] and exacerbated by the interactive effect of low [O_2] and pH (low pHOx). Under low pH stress, low [O_2] may impair the regulation of internal pH by embryonic squid, due to the reduced aerobic metabolic rate. This can lead to insufficient ATP production necessary to fuel active mechanisms for pH regulation and calcification. However, this indicator of an environmental response may not be useful as a proxy *per se*, because it likely tracks a sublethal effect on the embryo. Specifically, the uranium enrichment reflects the loss of pH regulation in the endolymph of the statocyst and may represent a threshold rather than a gradient.

The results presented show that the statolith chemistry records integrated the effects of the environment in concert with physiological processes, here identified as capsular effects (Tables 2 and 4).

These data suggest that statolith-chemical composition has a substantial disconnect from the external seawater environment (unlike foraminifera and their shells and some corals and their skeletons). Elemental composition measurements from the capsule jelly, perivitelline fluid [89] and within the endolymph fluid of the statocyst in addition to environmental measurements would help clarify the relationship between the environment and statolith geochemistry.

4. Conclusions

For the first time that we are aware, we demonstrated that environmental pH and [O_2] affect squid statolith geochemistry (uranium:calcium) and that statolith geochemistry is strongly affected by factors associated with the capsule (capsular effects). The only other known study that tested the effects of environmental pH on squid-embryo statolith geochemistry found that only ^{65}Zn significantly differed from an elemental suite that included ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg and ^{54}Mn [49]. Evidence that environmental tracers in squid statoliths can track seawater pH and [O_2] is especially useful, because uranium has been shown to be promising for understanding squid life history, migrations and habitat use [88,90,91].

However, we did not find strong evidence that environmental pH and [O_2] effects can be resolved for the use of statolith geochemistry as environmental proxies of pH and [O_2]. We did find strong capsular effects. The mechanism behind the capsular effect on statolith elemental incorporation is presumably due to a process that similarly affects all embryos of the capsule. Mechanisms include maternal transfer [53,73,83,84] and capsular and chorion membrane structural differences [49,50] among capsules. Less likely mechanisms include processes within the embryos [17–19,45,46] that are expressed similarly among embryos. These capsular effects are the first evidence (statolith chemistry) of strong physiological differences among the same cohort, and the importance of these differences for the persistence of the *D. opalescens* population is not known. Future applications might include the use of uranium:calcium as a geochemical marker tracking the initiation and duration of sublethal effects.

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recommendations are those of the authors and do not necessarily reflect the views of California Sea Grant, state agencies, NOAA, NSF or the U.S. Department of Commerce.

Author Contributions

Lisa A. Levin and Michael O. Navarro conceived and funded the research. Michael O. Navarro, Emily E. Bockmon and Lisa A. Levin designed the experiments. Michael O. Navarro, Jennifer P. Gonzalez, Christina A. Frieder and Emily E. Bockmon performed the experiments. Michael O. Navarro, Emily E. Bockmon and Christina A. Frieder analyzed the data. Michael O. Navarro wrote the paper, and Lisa A. Levin, Christina A. Frieder and Emily E. Bockmon edited and proofed the paper.

Appendix

Table A1. Seawater elemental concentrations measured in the laboratory experiments. Values are the average ± one standard error for samples taken once a week in each treatment (n = 5). No treatment effects were found for either Experiments 1 or 2.

Treatment (Tank)	B (ppm)	Mg (ppm)	Ca (ppm)	Sr (ppm)	Ba (ppb)	U (ppb)
Experiment 1						
Low pHOx (1)	4.45 ± 0.005	1092 ± 11.6	351.0 ± 3.40	5.64 ± 0.055	4.41 ± 0.120	1.98 ± 0.410
Low pHOx (2)	4.45 ± 0.005	1100 ± 14.8	353.2 ± 4.39	5.70 ± 0.084	4.51 ± 0.129	2.03 ± 0.408
High pHOx (1)	4.45 ± 0.003	1113 ± 12.6	357.7 ± 4.16	5.76 ± 0.064	4.65 ± 0.062	2.12 ± 0.428
High pHOx (2)	4.44 ± 0.014	1118 ± 14.7	358.5 ± 5.00	5.78 ± 0.074	4.51 ± 0.153	2.03 ± 0.404
Treatment Effect	$F_{1,4}$ = 1.588, p = 0.222	$F_{1,4}$ = 2.039, p = 0.169	$F_{1,4}$ = 1.969, p = 0.176	$F_{1,4}$ = 2.077, p = 0.165	$F_{1,4}$ = 0.957, p = 0.340	$F_{1,4}$ = 0.030, p = 0.864
Experiment 2						
Low [O_2] (1)	4.48 ± 0.004	1100 ± 14.9	355.6 ± 4.99	5.69 ± 0.077	4.34 ± 0.115	2.11 ± 0.190
Low [O_2] (2)	4.48 ± 0.004	1115 ± 15.1	358.6 ± 6.28	5.76 ± 0.089	4.51 ± 0.168	2.20 ± 0.176
Low pH (1)	4.48 ± 0.003	1131 ± 17.8	365.4 ± 6.10	5.87 ± 0.097	4.41 ± 0.067	2.08 ± 0.247
Low pH (2)	4.48 ± 0.004	1108 ± 31.7	359.2 ± 10.45	5.72 ± 0.163	4.34 ± 0.083	1.85 ± 0.122
Treatment Effect	$F_{1,4}$ = 0.038, p = 0.847	$F_{1,4}$ = 0.336, p = 0.571	$F_{1,4}$ = 0.513, p = 0.484	$F_{1,4}$ = 0.607, p = 0.447	$F_{1,4}$ = 0.189, p = 0.669	$F_{1,4}$ = 1.016, p = 0.329

Conflicts of Interest

The authors declare no conflict of interest.

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