Extreme low oxygen and decreased pH conditions naturally occur within developing squid egg capsules

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Running head: Extreme conditions in squid egg capsules

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Abstract

Young animals found future cohorts and populations but are often particularly susceptible to environmental changes. This raises concerns that future conditions, influenced by anthropogenic changes such as ocean acidification and increasing oxygen minimum zones, will greatly affect ecosystems by impacting developing larvae. Understanding the potential impacts requires addressing present tolerances and the current conditions in which animals develop. Here, we examined the changes in oxygen and pH adjacent to and within normally-developing squid egg capsules, providing the first observations that the egg capsules, housing hundreds of embryos, had extremely low internal pH (7.34) and oxygen concentrations (1.9 μmol L^{-1}). While early-stage egg capsules had pH and oxygen levels significantly lower than the surrounding seawater, late-stage capsules dropped dramatically to levels considered metabolically stressful even for adults. The structure of squid egg capsules resulted in a closely packed unit of respiring embryos, which likely contributed to the oxygen-poor and CO_2-rich local environment. These conditions rivaled the extremes found in the squids' natural environment, suggesting they may already be near their metabolic limit and that these conditions may induce a hatching cue. While squid may be adapted to these conditions, further climate change could place young, keystone squid outside of their physiological limits.

Introduction

Shifts in oceanic chemistry, such as changes in available oxygen (O_2) and decreasing pH, are of growing concern given their potential impacts on marine organisms and the ecosystems they support (Pörtner et al. 2004, Seibel and Childress 2013, Rosa et al. 2014). Dissolved O_2 is necessary for cellular respiration, but in many oceanic regions O_2 levels are declining and oxygen minimum zones are expanding (Stramma et al. 2012). These changes are attributed to several factors including lower sea-surface O_2 concentrations, local eutrophication events, and reduced ventilation from ocean warming. Decreasing O_2 levels are compounded by increasing carbon dioxide (CO_2) concentrations, largely from fossil fuel burning, which drive ocean acidification (Caldeira &Wickett 2003). Despite concerns regarding future ocean conditions, the baseline environmental conditions organisms currently face still requires substantial attention, especially in highly dynamic coastal ecosystems where parameters such as O_2 and pH fluctuate dramatically (Gobler et al. 2014, Wallace et al. 2014). Consequently, there are large uncertainties when predicting the influence of current and future environmental changes on key marine taxa. Baseline data affords a better understanding of these animals' current tolerances and contributes to the reduction of these uncertainties.

In nearly all marine environments, young, developing animals appear particularly susceptible to changing ocean conditions, with effects such as impaired development and reduced size having been shown for a variety of species (Kurihara 2008, Ries et al. 2009, Rosa et al. 2013). These impacts have been suggested to reduce recruitment success and, consequently, could reduce population abundances (Munday et al. 2010). Marine invertebrates that deposit calcareous skeletons have received much attention with their young showing vital changes (i.e., growth, structure) when raised under ocean acidification or hypoxic conditions (Hoegh-Guldberg et al. 2007, Ries et al. 2009, Gobler et al. 2014). Impacts to soft-bodied invertebrates are seemingly less understood, yet they too have calcified structures, and are often physiologically limited by pH, aragonite concentrations, and O_2 levels (Radtke 1983, O'Dor et al. 1994, Pörtner et al. 2004, Rosa et al. 2013). Any mechanism that may even slightly reduce the early-life survival rates of marine organisms can have major repercussions on adult population sizes and, in the case of keystone taxa, overall ecosystem health (Houde 1987, 2008).

Cephalopods, particularly squid, are an ecologically and economically key taxon, providing a central trophic link in many marine food webs and 15-20% of global fisheries landings and values (Boyle & Rodhouse 2005, Hunsicker et al. 2010). The Loliginid squid are the primary commercial cephalopod of the western North Atlantic and support a fishery of approximately 16,600 mt yr^{-1} (NOAA 2010). Occasionally cited as keystone taxa, squid play a central role in food webs as predator and prey to a wide array of taxa that occupy different trophic levels (Clarke 1996). Cephalopods are no exception to the potential impacts of changing ocean conditions. Increased pCO_2 can cause significant increases in development time, decreases in hatchling size, and changes to statolith structure in squid (*D. pealeii)*(Kaplan et al. 2013). In adults, decreased pH can impair the O_2 binding capacity of haemocyanin, the squid respiratory protein (Pörtner 1990). Even in today's oceans, adult squid are considered to live near the edge of O_2 limitation, particularly as they exercise (Pörtner et al. 1991, Seibel 2007, Seibel & Childress 2013). Hence, lower metabolic rates occur across different squid taxa as respective environmental O_2 concentrations decrease (Seibel 1997). Small decreases in ambient pH or O_2

are thought to endanger haemocyanin's ability to bind sufficient O_2 or otherwise limit O_2 uptake, and would likely impair the squid's high energetic demand (Pörtner et al. 2004, Seibel & Childress 2013).

Squid recruitment is largely driven by environmental factors (Dawe et al. 1990) and environmental conditions play a large role in migrations, distribution, growth, and spawning (Boyle & Rodhouse 2005, Zeidberg et al. 2011). Because cephalopod abundances are directly tied to the success of early life history, growth, and survival (Boyle & Rodhouse 2005, Foote et al. 2006), environmental changes such as ocean acidification or decreased O_2 availability could directly impact populations. With growing evidence that ocean acidification will be amplified by hypoxia and eutrophication in coastal waters, it is becoming increasingly important to consider the interaction of these environmental parameters (Melzner et al. 2013, Cai et al. 2011, Wallace et al. 2014), particularly on the susceptible early life stages. Adjacent, near-shore estuarine habitats where squid such as *D. pealeii* are occasionally found may vary substantially in pH and O_2, conditions that are exacerbated by eutrophication (Wallace et al. 2014, Baumann et al. 2015). Yet, for *D. pealeii* and many other squid species there are few data addressing the epi-benthic, coastal environment (pH, O_2, flow) where the majority of reproductive adults are harvested, and thus, the conditions that most egg capsules naturally experience.

There is a growing body of literature that addresses O_2 availability or O_2 and pH conditions associated with developing mollusks (Booth 1995, Cohen & Strathmann 1996, Moran & Woods 2007). Work on cephalopods has largely focused on cuttlefish (Cronin & Seymour 2000, Gutowska & Melzner 2009, Dorey et al. 2013), a taxon in which a single embryo develops in individual capsules. Adult Loliginids, like many coastal squid, lay their eggs in gelatinous capsules on the benthos with each egg capsule densely housing 150-200 embryos (Hanlon & Messenger 1996). These animals undergo rapid growth, becoming fully developed in 12-14 days at 20° C (McMahon & Summers 1971), with the capsule expanding in size to accommodate this growth (Hanlon et al. 1983). Although most hatching occurs during the night and certain physical disturbances (such as handling) may induce hatching (Hanlon & Messenger 1998, Zeidberg et al. 2011), the natural cues or catalysts for squid egg hatching (besides full development) are not well established. While recent work has considered the respiration of squid embryos, individuals were removed from their capsules for respirometry measurements creating a design unlike that of nature, and potentially promoting premature stress and hatching (Rosa et al. 2012, Rosa et al. 2014). Thus, to date, squid embryos consume an unknown amount of O_2 and produce an unquantified amount of CO_2, all within a semi-permeable capsule, the structure of which likely alters the exchange of O_2 and CO_2. Further, it remains poorly understood how a population of fast growing, highly active cephalopod embryos within a capsule influences pH and O_2 within the capsule or adjacent water, and how this influence may vary with development, embryo size, and increases in O_2 demand and CO_2 respiration. Such studies would require a detailed profile of the egg capsule and the surrounding physical boundary layer of intact capsules as has been done with metabolically slower gastropods and polychaetes (Chafee & Strathmann 1984, Booth 1995, Cohen & Strathmann 1996, Moran & Woods 2007). For example, in many marine gastropods which lay benthic egg clutches, intracapsular O_2 availability substantially affects embryo development rates (Booth 1995, Strathmann & Strathmann 1995). Local environmental conditions can also affect oxygen uptake and consequent embryo condition (Cohen & Strathmann 1996, Cancino et al. 2011). Further, the physical boundary layer

surrounding egg capsules is a function of the physical characteristics of water flow rates and the roughness of the capsule surface and may significantly alter exchange across organismal boundary layers. The variation of this boundary layer due to fluctuating flow rates in coastal ecosystems has not been considered in experiments of changing ocean conditions or metabolism on cephalopod egg capsules.

To address these unknowns and provide a better understanding the natural pH and O_2 conditions associated with a densely populated cephalopod egg capsule, this work sought to quantify (1) the O_2 and pH levels within egg capsules where embryos develop, (2) the egg capsule O_2 consumption and pH change across embryonic development, and (3) the pH and O_2 in the boundary layer adjacent to the capsule. These data were then placed in the context of current data on thresholds and pH and O_2 limits for squid, to highlight what data are needed for this critical developmental stage.

Methods

Experiments were conducted at the Woods Hole Oceanographic Institution (WHOI), MA in August-September, 2014. The collection of adult squid was conducted under Massachusetts Division of Marine Fisheries research permit #152087. Husbandry and animal care were performed in accordance with guidelines as approved by WHOI's Institutional Animal Care and Utilization Committee. Squid, *D. pealeii*, were trawl-caught in Vineyard Sound, MA, USA on two occasions in 10-20 meters of water. Adults in healthy condition (free of cuts and scrapes) were hand-selected from the group, gently placed in individual buckets, and were immediately transported to a 500L holding tank at WHOI and maintained in 14°C cooled, filtered, flowing local water. Within ~48-72h the squid bred, laying eggs capsules in a mass on the tank bottom. Egg masses (~30 capsules $mass^{-1}$) were transferred to either a 38L aquarium in which water was replaced daily, or a 100L flow-through aquarium, both of which were filled with local filtered-seawater maintained at 20ºC, which is the average temperature for Vineyard Sound during the study period (19.4 ± 0.68 (± SD), data from Martha's Vineyard Coastal Observatory, http://www.whoi.edu/page.do?pid=70177). Individual egg capsules were separated from the egg mass immediately prior to profiling, attached on top of 5mm rigid plastic mesh using zip ties at the leading edge of the capsule, and transferred to a 0.5L glass container or a custom 9.5L recirculating micro–flume, both filled with the same filtered 20ºC seawater. All measurements and incubations where done under ambient laboratory light conditions. A micromanipulator (Unisense, DK) was used to vertically profile up to and within the egg capsules (Fig 1).

A FireStingO_2 optical oxygen sensor (50µm sensing tip) and meter (Pyroscience, GE) and liquid ion exchange (LIX) pH sensors (5-20µm sensing tip) were used to measure profiles. LIX pH sensors were constructed and used following (Gieseke & de Beer 2004). To describe briefly, glass capillaries were pulled to a tip diameter of 5-20 µm and the glass was silinized using N,N-dimethyltrimethylsilylamine (Sigma, USA) in a sealed glass container at 200 ºC to make the glass hydrophobic. Poly-vinyl chloride stabilized H^+ sensitive membranes (H^+ ionophore II, Sigma, USA) were pulled into the capillary tips, and the electrode was back-filled with a 300 mmol L^{-1} potassium chloride, 7.0 pH, 50 mmol L^{-1} phosphate buffer. The micro electrodes were finished by sealing a silver chloride plated 0.25 mm diameter silver wire into the back of the capillary. The electrochemical circuit was completed with a reference electrode

consisting of a glass capillary filled with a saturated potassium chloride solution, a microporous glass frit (Princeton Applied Research, USA) tip, and sealed with a silver chloride plated 0.25 mm diameter silver wire. The millivolt response of the electrodes (> 50 mV per pH unit) was measured using a high-impedance millivolt meter.

The pH sensors were calibrated using NIST-traceable buffers at 20 ºC and cross-checked before and after each profile with a commercial pH sensor (Hach, USA). The Pyroscience O_2 optodes were calibrated using a saturated sodium ascorbate solution (0% O_2) and water-saturated air according to manufacturer instructions. To ensure sensors were not damaged or otherwise affected by profiling through the egg capsules, sensors were returned to the ambient water after reaching the center of the egg capsules to confirm that the sensors showed consistent readings with the beginning of the profile

The recirculating mini-flume consisted of a divided 9.5L aquaria connected by a passage 0.07 high, 0.07 wide, and 0.3 m long. Water was pumped between the aquaria halves using a small pump and the flow adjusted using a ball-valve. Flow rates were evaluated with simple discharge-area-time relationships. Sensors were located using the micromanipulator and a forward-looking adjustable (0-25x) dissection scope (Zeiss, GE), all mounted to a sturdy microprofiling base station (Unisense, DK). The mini-flume water was changed daily using the filtered, local seawater at 20 ºC. Static (no-flow) profiles were determined in 0.5L glass containers within the same microprofiling base station, and filled with water from the aquaria the capsules were incubated in. Egg capsules were incubated in the flume at a specific flow rate for at least 1 hour prior to profiling.

A coarse vertical profile (1000 µm increments) was measured in the water column down to the capsule "boundary layer", or the fluid layer around the egg capsule where diffusive transport is of primary importance, defined here by the location where large O_2 concentration changes were observed (e.g. Figure 1, Gieseke & de Beer 2004). At the boundary layer measurement increments were decreased to 100 µm to better resolve the concentration gradient. The sensors were then pushed into the egg capsule and the profile continued until reaching the egg capsule center, determined using a micrometer in the dissection scope, the egg capsule diameter, and the visible location of the sensor tip. Care was taken to prevent the puncture of individual embryos; the small sensor movements allowed the embryos to shift laterally, allowing the sensors to remain within the intracapsular fluid. Each profile was done on a new egg capsule in the range of 1-3 or 10-13 days-old. Differences between the ambient conditions and conditions in the center of the different aged capsules were determined by one-way ANOVAs with differences between these groups determined by Tukey post-test (Figure 2, Table 1). Due to the seasonal cessation of squid breeding, testing of flow effects on egg capsule profiles could only be conducted with unfertilized egg capsules. However, these provided initial baseline profiles of egg capsule respiration due to microbial biomass (i.e., no metabolism of developing embryos) and the effects of water flow past capsules at low (0.01 $m \cdot s^{-1}$) and high (0.1 $m \cdot s^{-1}$) current velocities utilizing the recirculating micro-flume.

Results

Newly-laid egg capsules demonstrated significantly lower O_2 concentrations and pH relative to the ambient water, with O_2 dropping from ~200 µmol L^{-1} O_2 to 160 at the capsule

center and a pH decrease from 8.0 to 7.8 (Figure 1A, C). This difference increased substantially with egg development (Figure 1B, C). After 10-13 days of development the egg capsule centers contained only trace amounts of O_2 (1.9 ±1.1 µmol L^{-1}) and had a pH of 7.34 ±0.01 (±SD).

The steep gradients between the ambient water and the egg capsule allowed for the calculation of diffusive O_2 flux across the boundary layer around the egg capsule (Figure 1). The dominant transport process in this boundary layer is diffusion; therefore Fick's Law of Diffusion can be used to determine exchange across the egg capsule surface. The flux = $\partial O_2/\partial x * D$, where D is the diffusion coefficient of O_2, and x is the depth (Gieseke & de Beer 2004). The depth of the boundary layer (x) was determined from the concentration profiles and the O_2 gradient was determined from the O_2 gradient between the ambient water and the egg capsule surface. The fluxes revealed a 10-fold increase in egg capsule O_2 consumption over a 10-day period (0.060 to 0.595 µmol cm^{-2} min^{-1} for the 1-3 and 10-13 day-old capsules, respectively). Applying Fick's Law of Diffusion to the capsule boundary layer allows for the determination of the time point when the maximum physical transport into the capsule is exceeded by the capsule metabolic requirement (indicating significant hypoxic stress). For example, using the measured boundary layer thickness, the maximum possible O_2 gradient (~200 µmol L^{-1} mm^{-1}), and assuming a linear increase in O_2 consumption with capsule age, the time when the maximum physical transport of O_2 is exceeded by egg O_2 consumption (0.84 µmol cm^{-2} min^{-1}) was 15.8 days.

The unfertilized egg capsules had similar O_2 concentration changes and profiles to the 1-3 day-old egg capsules (Figure 2; Table 1). The former is primarily due to the metabolism of capsule-associated microbial communities (Barbieri et al. 2001) and suggests a relatively small measureable metabolic contribution by the 1-3 day-old embryos. The calculated O_2 fluxes were 0.073, 0.088, and 0.098 µmol cm^{-2} min^{-1} for unfertilized egg capsules under no-flow, low-flow, and high-flow conditions, respectively. The thickness of the boundary layer decreased with flow (2.0, 0.7, and 0.4mm for the no-flow, low-flow, and high-flow conditions, respectively).

All of the egg capsules used in this study hatched viable squid paralarvae, with the exception of the unfertilized capsules (characterized by no change in size or visible growth). Hatching success was not evaluated as a part of this work. The developing egg capsules visibly increased in volume as the embryos grew (Table 1), leading to the deeper profiles in the 10-13 day-old egg capsules.

Discussion

Conditions in the full-term embryo capsules were unexpectedly low in both O_2 and pH, reaching levels that are often considered adverse to many pelagic taxa (Stramma et al. 2012). These O_2 levels were below that of water adjacent to the capsules and that of the local environment where these capsules are often found, as well as even that of Atlantic oxygen minimum zones (Karstensen et al. 2008). Although some oceanic deep-sea squid species have shown tolerances and even affinities to low O_2 levels (Gilly et al. 2012, Seibel 2013), active, coastal, adult Loliginid squids are typically considered near the edge of metabolic O_2 capabilities and somewhat intolerant of the conditions measured here (Pörtner 2002). The decrease of O_2 levels by 99% to near-anoxic conditions across growth is larger than the hypoxic conditions observed for similar cephalopod species that have a single egg per capsule (75% decrease (Dorey

et al. 2013), 86% decrease (Cronin and Seymour 2000), 62% decrease (Gutowska and Melzner 2009), 85% decrease (Rosa et al. 2013)) suggesting the densely packed egg capsule structure leads to a an extremely high O_2 demand, as has been found in a number of gastropod and polychaete species housing multiple eggs per capsule (Chafee & Strathmann 1984, Booth 1995, Cohen & Strahmann 1996, Moran & Woods 2007). The observation of hatching and healthy paralarvae was surprising based on previous results from studies on cephalopods, and suggests these conditions may not have induced extreme stress, similar to results found in other multiple-egg per capsule species.

The boundary effects of the capsule suggest that encapsulation of the many embryos likely contributes to the lower O_2 and pH (Chafee & Strathmann 1984, Booth 1995, Moran & Woods 2007), resulting in conditions that are substantially lower than observed for single-egg-per-capsule species (e.g., cuttlefish (Rosa et al. 2013)). The encapsulation of embryos has been proposed as a mechanism to protect embryos against ocean acidification through the buffering capacity of intracapsular fluids (Ellis et al. 2009, Fernandes & Podolsky 2012) but our data suggest that encapsulation causes reduced pH conditions around embryos. We expect even lower pH and O_2 values inside the egg capsules of squid raised in elevated ocean acidification or low oxygen conditions (as seen in some taxa (Rosa et al. 2013, Noisette et al. 2014)). However, it is not clear whether these lower pH or O_2 conditions would lead to greater impacts or perhaps support adaptation to future, changing conditions.

Squid, particularly muscular, shallower species such as the taxa studied here, are considered relatively intolerant to small changes in pH (Pörtner et al. 2004). The blood pH for these adult squid is typically near 7.6 (Pörtner 1990) with some exceptions for specialized species living in OMZ or the deep ocean (Seibel 2013, Seibel and Childress 2013). The intracapsular levels of pH 7.34 noted here were unexpected for such energetic coastal squid and were also well below environmental levels which have induced developmental changes in young squid (Kaplan et al. 2013, Rosa et al. 2014). This implies that, during prior studies, pH values inside the experimental capsules (Kaplan et al. 2013, Rosa et al. 2014) were even lower, perhaps suggesting that despite these extreme conditions, young squid may be more tolerant than previously considered. Further, the measured levels of this study were near the limit of predicted pH-dependent blood pigment O_2 affinity (Pörtner 1990); at a lower pH, blood might not effectively take up O_2. These embryos may already be at relatively inefficient metabolic levels due to the combined effect of low pH and O_2 or their haemocyanin pigment may have an improved affinity compared to adults, for example, due to the presence of different isoforms of haemocyanin in cephalopod embryos that may be more efficient at O_2 binding (Thonig et al. 2014). However, the higher surface area-to-volume ratio and importance of cutaneous O_2 uptake in squid embryos indicates that pigment-mediated O_2 exchange would likely be less important. While pH is a likely stressor for many squid, the low O_2 concentrations may dominate in limiting metabolism and energetics (Seibel et al. 1997, Seibel & Childress 2013). Thus, the low O_2 levels of egg capsules seen here reinforce concerns of expanding OMZs and deoxygenation of ocean waters, particularly if the limits of organismal adaptation are reached.

Certainly these squid may be adaptable to changes, as seen with tolerance to low O_2 conditions in the young of some more specialized squid species (Seibel 2013, Trübenbach et al. 2013, Seibel et al. 1997). While the conditions shown here are relatively extreme for the open

ocean, they may be less stressful for coastal and estuarine organisms, which may have a greater range of tolerances (Murray et al. 2014). Based on data from adult squid and multiple other taxa (Pörtner et al. 2004, Seibel and Childress 2013), these squid may already be near a physiological limit, or less adaptable in the face of future ocean biogeochemical changes. Neither the optimal nor threshold O_2 and pH levels for embryonic development have been defined, so we do not know whether these conditions place substantial stress on the developing squid. The effect of the low intracapsular pH and O_2 observed here requires further study to determine how squid may be affected by future O_2 and CO_2 conditions. Understanding the mechanisms and the potential for developing squid embryos to withstand these conditions will inform our expectations for how these and other organisms may cope with projected global ocean changes.

Both pH and O_2 levels decreased over time, reflecting the increased size and energetic demand of developing squid. As shown elsewhere (Pörtner et al. 2004, Seibel and Childress 2013), there are limits to squid pH and oxygen tolerances; we suggest here that these levels may even act as an embryonic hatching cue. The maximum time to hatching was estimated from the maximum physical O_2 gradient across the egg capsule boundary layer (Figure 2D). This maximum exchange rate under no-flow conditions suggests a maximum hatching time of 15.8 days under the observed conditions, which is consistent with previously observed hatching times of 12-14 days (Kaplan et al. 2013). The decrease to such low levels within the capsule may act as an embryonic hatching cue, and compounding O_2 and pH changes in the surrounding environment may induce premature hatching. Water flow over the capsule likely plays a role as the capsule boundary layer thickness decreased with increasing flow (Figure 2C) and O_2 exchange increased in the unfertilized egg capsules suggesting enhanced exchange across the capsule surface due to current flow. Previously, hatching has been observed at night and explained as a mechanism to reduce being eaten by visual predators (Zeidberg et al. 2011). Conversely, hatching may be induced during hydrodynamically calm periods during the night when O_2 is reduced by ecosystem respiration and the absence of photosynthesis, for example, during a nighttime slack tide. The enhanced exchange with flow also suggests that egg laying locations that have active hydrodynamics may lead to faster embryonic development and reduced stress by low pH and O_2 conditions (Chafee & Strathmann 1984, Cohen & Strahmann 1996, Zeidberg et al. 2011). Therefore, ocean acidification and hypoxia experiments should consider the effects of flow-enhanced exchange between organisms and the ambient seawater.

References

Barbieri E, Paster B, Hughes D, Zurek L, Moser D, Teske A, Sogin M (2001) Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid Loligo pealei (*Cephalopoda:Loliginidae*). Environmental Microbiology 3:151-167

Baumann H, Talmage SC, Gobler CJ (2012) Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nature Climate Change 2:38-41

Baumann H, Wallace RB, Tagliaferri T, Gobler CJ (2015) Large natural pH, CO2 and O2 fluctuations in a temperate tidal salt marsh on diel, seasonal, and interannual time scales. Estuaries and Coasts 38: 220-231

366 Boyle P, Rodhouse P (2005) Cephalopods: Ecology and fisheries, Vol. Blackwell Science,
367 Oxford, UK
368 Booth D (1995) Oxygen availability and embryonic development in sand snail egg masses. J
369 Exper Biol198: 241-247.
370 Cai WJ, Hu X, Huang WJ, Murrell MC, Lehrter JC, Lohrenz SE, Zhao P (2011) Acidification of
371 subsurface coastal waters enhanced by eutrophication. Nature Geoscience 4: 766-770.
372 Cancino JM, Gallardo JA, Brante A (2011) The relationship between temperature, oxygen
373 condition and embryo encapsulation in the marine gastropod chorus giganteus. Journal of
374 the Marine Biological Association of the United Kingdom 91:727-733
375 Chaffee C, Strathmann RR (1984) Constraints on egg masses. I. Retarded development within
376 thick egg masses. Journal of Experimental Marine Biology and Ecology, 84: 73-83
377 Clarke MR (1996) Cephalopods as prey. III. Cetaceans. Philos T Roy Soc B 351:1053-1056
378 Cohen CS, Strathmann RR (1996) Embryos at the edge of tolerance: effects of environment and
379 structure of egg masses on supply of oxygen to embryos. The Biological Bulletin, 190: 8-
380 15
381 Cronin ER, Seymour RS (2000) Respiration of the eggs of the giant cuttlefish Sepia apama. Mar
382 Biol 136: 863-870
383 Dawe EG, Shears JC, Balch NE, O'Dor RK (1990) Occurrence, size and sexual maturity of long-
384 finned squid (*Loligo pealei*) at Nova Scotia and Newfoundland, Canada. Can J Fish Aqua
385 Sci 47:1830-1835
386 Dorey N, Melzner F, Martin S, Oberhänsli F, Teyssié JL, Bustamante P, Lacoue-Labarthe T
387 (2013. Ocean acidification and temperature rise: effects on calcification during early
388 development of the cuttlefish Sepia officinalis. Mar Biol 160: 2007-2022
389 Ellis R, Bersey J, Rundle S, Hall-Spencer J, Spicer J (2009) Subtle but significant effects of CO2
390 acidified seawater on embryos of the intertidal snail, Littorina obtusata. Aqua Biol 5:41-
391 48
392 Fernandes D, Podolsky R (2012) Effects of ocean acidification on growth, development, and
393 calcification of gastropod embryos: does encapsulation matter? . Integrative and
394 comparative biology 52:e244-e244
395 Foote KG, Hanlon RT, Iampietro PJ, Kvitek RG (2006) Acoustic detection and quantification of
396 benthic egg beds of the squid *Loligo opalescens* in Monterey Bay, California J Acoust
397 Soc Am 119:844-856
398 Gieseke A, de Beer D (2004) Use of microelectrodes to measure in situ microbial activities in
399 biofilms, sediments, and microbial mats, Vol. Kluwer, Molecular Microbial Ecology
400 Manual
401 Gilly WF, Zeidberg LD, Booth JAT, Stewart JS, Marshall G, Abernathy K, Bell LE (2012)
402 Locomotion and behavior of Humboldt squid, *Dosidicus gigas*, in relation to natural
403 hypoxia in the Gulf of California, Mexico. J Exp Biol 215:3175-3190
404 Gobler CJ, DePasquale EL, Griffith AW, Baumann H (2014) Hypoxia and acidification have
405 additive and synergistic negative effects on the growth, survival, and metamorphosis of
406 early life stage bivalves. PloS one 9:e83648
407 Gutowska MA, Melzner F (2009) Abiotic conditions in cephalopod (Sepia officinalis) eggs:
408 embryonic development at low pH and high pCO2. Mar Bbiol 156: 515-519
409 Hanlon R, Messenger JB (1996) Cephalopod behavior, Vol. Cambridge University Press, New
410 York

411 Hanlon R, Messenger JB (1998) Cephalopod behavior, Vol. Cambridge University Press, New
412 York
413 Hanlon RT, Hixon RF, Hulet WH (1983) Survival, growth and behavior of the loliginid squids,
414 *Loligo plei*, *Loligo pealei* and *Lolliguncula brevis* (Mollusca: Cephalopoda) in closed
415 seawater systems. Biol Bull 165:637-685
416 Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD,
417 Sale PF, Edwards AJ, Caldiera K, Knowlton N, Eakin M, Inglesias-Prieto R, Muthiga N,
418 Bradbury RH, Dubi A, Hatziolos ME (2007) Coral Reefs Under Rapid Climate Change
419 and Ocean Acidification. Science 318:1737-1742
420 Houde ED (1987) Fish early life dynamics and recruitment variability. In: Hoyt RD (ed)
421 American Fisheries Society Symposium 2. American Fisheries Society, Bethesda, MD, p
422 17-29
423 Houde ED (2008) Emerging from Hjort's shadow. J Northw Atl Fish Sci 41:53-70
424 Hunsicker ME, Essington TE, Watson R, Sumaila UR (2010) The contribution of cephalopods to
425 global marine fisheries: can we have our squid and eat them too? Fish and Fisheries
426 11:421–438
427 Kaplan MB, Mooney TA, McCorkle DM, Cohen A (2013) Adverse effects of ocean acidification
428 on early development of squid (*Doryteuthis pealeii*). PLoS ONE 8:e63714
429 Karstensen J, Stramma L, Visbeck M (2008) Oxygen minimum zones in the eastern tropical
430 Atlantic and Pacific oceans. Progress in Oceanography 77:331-350
431 Kurihara H (2008) Effects of CO2-driven ocean acidification on the early developmental stages
432 of invertebrates. Marine Ecology Progress Series 373:275-284
433 McMahon JJ, Summers WC (1971) Temperature effects on the developmental rate of squid
434 (*Loligo pealei*) embryos. Biol Bullet 141:561-567
435 Melzner F, Thomsen J, Koeve W, Oschlies A, Gutowska MA, Bange HW, Körtzinger A (2013)
436 Future ocean acidification will be amplified by hypoxia in coastal habitats. Marine
437 Biology 160: 1875-1888
438 Moran AL, Woods HA (2007) Oxygen in egg masses: interactive effects of temperature, age,
439 and egg-mass morphology on oxygen supply to embryos. J Exper Biol 210: 722-731
440 Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchetta MS, Devitsin GV, Døving KB
441 (2009) Ocean acidification impairs olfactory discrimination and homing ability of a
442 marine fish. Proc Natl Acad Sci 106:1848-1852
443 Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MC, Chivers DP (2010)
444 Replenishment of fish populations is threatened by ocean acidification. Proc Natl Acad
445 Sci 107:12930-12934
446 Murray CS, Malvezzi A, Gobler CJ, Baumann H (2014) Offspring sensitivity to ocean
447 acidification changes seasonally in a coastal marine fish. Mar Ecol Prog Ser 504:1-11
448 NOAA (2010) 51st Northeast Regional Stock Assessment Review. Stock Assessment Review
449 Committee:54
450 Noisette F, Comtet T, Legrand E, Bordeyne F, Davoult D, Martin S (2014) Does Encapsulation
451 Protect Embryos from the Effects of Ocean Acidification? The Example of Crepidula
452 fornicata. PloS one 9:e93021
453 O'Dor RK, Hoar JA, Webber DM, Carey FG, Tanaka S, Martins H, Porteiro FM (1994) Squid
454 (*Loligo forbesi*) performance and metabolic rates in nature. Mar Freshw Behav Phy
455 25:163-177

Pörtner H-O (1990) An analysis of the effects of pH on oxygen binding by squid (*Illex illecebrosus, Loligo pealei*) haemocyanin. J Exp Biol 150:407-424
Pörtner H, Langenbuch M, Reipschläger A (2004) Biological Impact of Elevated Ocean CO2 Concentrations: Lessons from Animal Physiology and Earth History. J Ocean 60
Pörtner HO (2002) Environmental and functional limits to muscular exercise and body size in marine invertebrate athletes. Comp Biochem Physiol A 133:303-321
Pörtner HO, Webber DM, Boutilier RG, O'Dor RK (1991) Acid-base regulation in exercising squid (*Illex illedebrosus, Loligo pealei*). American Journal of Physiology 261:R239-R246
Radtke RL (1983) Chemical and structural characteristics of statoliths from the short-finned squid Illex illecebrosus. Marine Biology 76:47-54
Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology 37:1131-1134
Rosa R, Pimentel MS, Boavida-Portugal J, Teixeira T, Trübenbach K, Diniz M (2012) Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. PLoS One 7:e38282
Rosa R, Trübenbach K, Pimentel MS, Boavida-Portugal J, Faleiro F, Baptista M, Dionísio G, Calado R, Pörtner HO, Repolho T (2014) Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). J Exp Biol 217:518-525
Rosa R, Trübenbach K, Repolho T, Pimentel M, Faleiro F, Boavida-Portugal J, Baptista M, Lopes VM, Dionísio G, Leal MC (2013) Lower hypoxia thresholds of cuttlefish early life stages living in a warm acidified ocean. Proceedings of the Royal Society B: Biological Sciences 280:20131695
Seibel BA (2007) On the depth and scale of metabolic rate variation: Scaling of oxygen consumption rates and enzymatic activity in the class cephalopoda (mollusca). J Exp Biol 210:1-11
Seibel BA (2013) The jumbo squid, Dosidicus gigas (Ommastrephidae), living in oxygen minimum zones II: Blood–oxygen binding. Deep Sea Research Part II: Topical Studies in Oceanography 95: 139-144
Seibel BA, Childress JJ (2013) The real limits to marine life: a further critique of the Respiration Index. Biogeosciences 10: 2815-2819
Seibel BA, Thuesen EV, Childress JJ, Gorodezky LA (1997) Decline in pelagic cephalopod metabolism with habitat depth reflects differences in locomotory efficiency. The Biological Bulletin 192:262-278
Stramma L, Prince ED, Schmidtko S, Luo J, Hoolihan JP, Visbeck M, R.Wallace DW, Brandt P, Körtzinger A (2012) Expansion of oxygen minimum zones may reduce available habitat for tropical pelagic fishes. Nat Clim Chg 2:33-37
Strathmann RR, Strathmann MF (1995) Oxygen supply and limits on aggregation of embryos. Journal of the Marine Biological Association of the United Kingdom 75:413-428
Thonig A, Oellermann M, Loeb B, Mark FC (2014) A new haemocyanin in cuttlefish (*Sepia officinalis*) eggs: sequence analysis and relevance during ontogeny. EvoDevo 5:1-13
Trübenbach K, Pegado MR, Seibel BA, Rosa R (2013) Ventilation rates and activity levels of juvenile jumbo squid under metabolic suppression in the oxygen minimum zone. J Exp Biol 216:359-368
Wallace RB, Baumann H, Grear JS, Aller RC, Gobler CJ (2014) Coastal ocean acidification: The other eutrophication problem. Estuarine, Coastal and Shelf Science 148:1-13

Zeidberg LD, Isaac G, Widmer CL, Neumeister H, Gilly WF (2011) Egg capsule hatch rate and incubation duration of the California market squid, Doryteuthis (= Loligo) opalescens: insights from laboratory manipulations. Marine Ecology 32:468-479

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Table 1. Oxygen and pH in the ambient water and center of egg capsules

	Egg Capsule Diameter	**Oxygen**	**pH**
	(mm)	(μmol L^{-1})	(units)
Ambient Seawater	-	198.5 ± 5.5^{a} (*3*)	8.01 ± 0.01^{a} (*6*)
Unfertilized egg capsules	6.53 ± 0.30^{a} (*3*)	169.3 ± 0.0^{ab} (*1*)	7.86 ± 0.03^{b} (*2*)
1-3 day old egg capsules	5.22 ± 0.28^{a} (*5*)	168.3 ± 12.8^{b} (*2*)	7.80 ± 0.01^{c} (*3*)
10-13 day old egg capsules	13.7 ± 1.86^{c} (*5*)	1.9 ± 1.1^{c} (*2*)	7.34 ± 0.01^{d} (*3*)
F_3	69.6013	299.6569	1640.154
p	< 0.0001	< 0.0001	< 0.0001

The *F* and *p* values indicate significant differences determined by ANOVAs. Superscript letters indicate significant differences between groups by Tukey post-tests. The (*n*) is the number of egg capsules.

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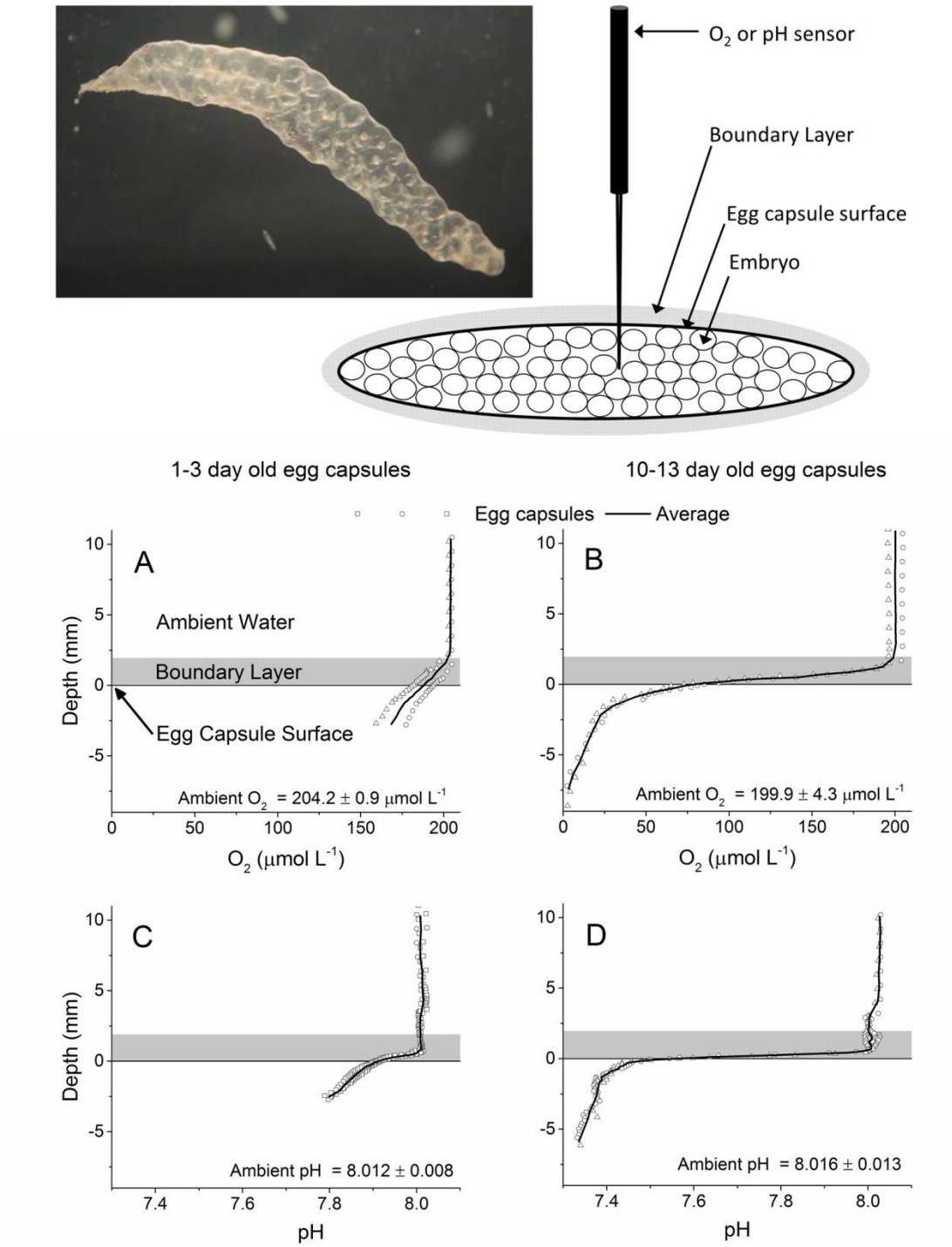

Figure 1. Egg capsule picture and schematic of profiling, boundary layer (grey shading), and egg capsule (top). Profiles of oxygen (A,B) and pH (C,D) in 1-3 day-old (left) and 10-13 day-old (right) egg capsules. Shapes indicate individual profiles in different egg capsules and the solid lines are the average profile. Photo credit: C. Zakroff.

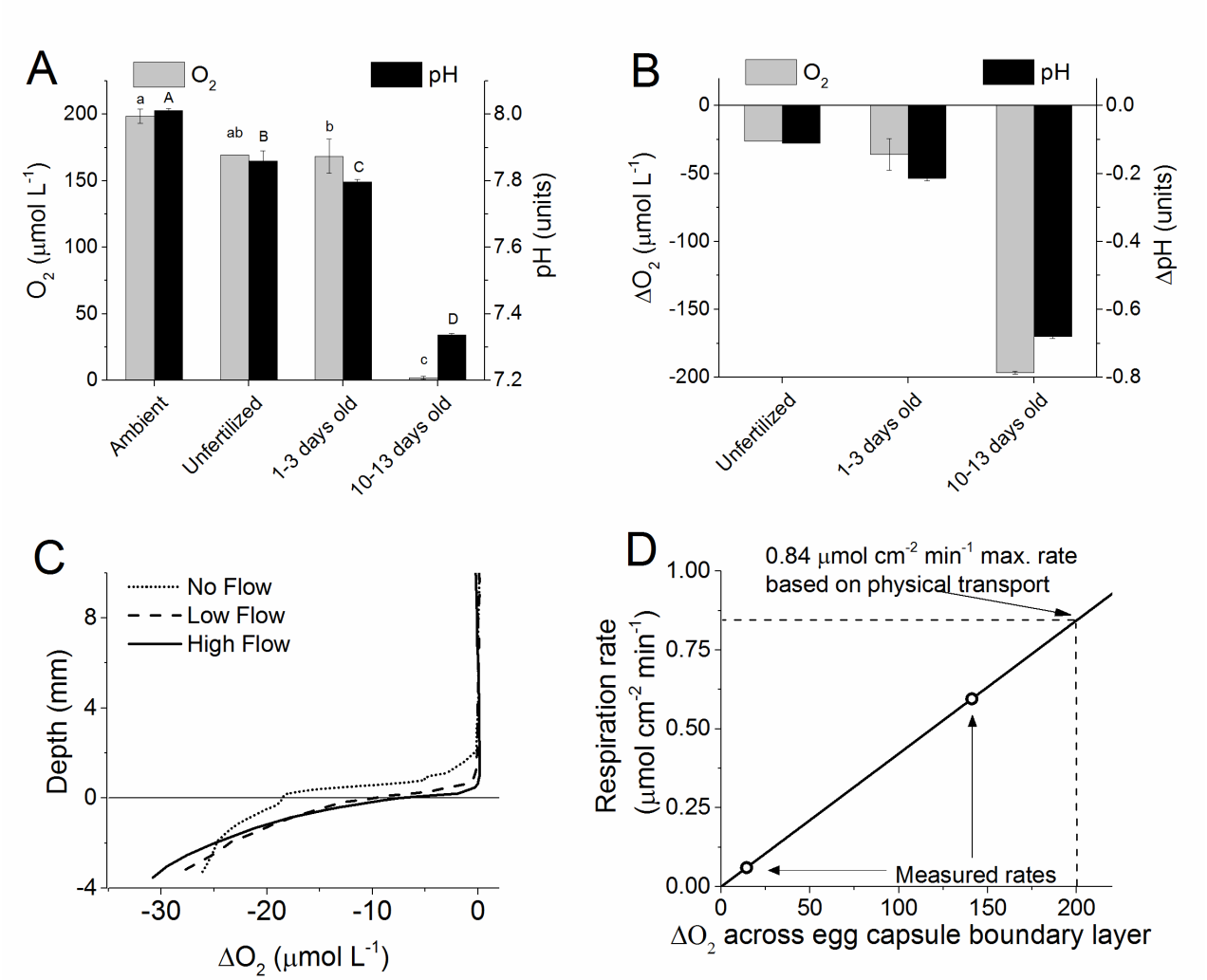

Figure 2. Oxygen concentrations and pH (A) in ambient water and the center of different aged egg capsules showing significant variation in both oxygen and pH (Table 1); letters indicate differences between groups (using Tukey post-tests). (B) The change in oxygen and pH in the center of egg capsules relative to the ambient water conditions. (C) The effect of different flow rates on oxygen profiles in unfertilized egg capsules and the compression of the boundary layer where each line represents the average of three profiles. (D) The maximum respiration rate under no-flow conditions (see text) indicating egg capsules ~16 days-old will be significantly stressed during hydrodynamically calm periods.