

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

3
4 Matthew H. Long^{1*}, T. Aran Mooney^{2*}, and Casey Zakroff^{2,3}

5
6
7 ¹Marine Chemistry and Geochemistry Department, Woods Hole Oceanographic Institution, 266
8 Woods Hole Road, Woods Hole, Massachusetts, USA 02543

9
10 ²Biology Department, Woods Hole Oceanographic Institution, 266 Woods Hole Road, Woods
11 Hole, Massachusetts, USA 02543

12
13 ³Massachusetts Institute of Technology-Woods Hole Oceanographic Institution Joint Program in
14 Oceanography/Applied Ocean Science and Engineering, Cambridge, Massachusetts, USA 02139

15
16
17 *contact: mlong@whoi.edu; amooney@whoi.edu

18
19
20 Running head: Extreme conditions in squid egg capsules

21
22 Keywords: cephalopod, climate change, hypoxia, boundary layer, eggs, larva

23
24
25
26 **Abstract**

27
28 Young animals found future cohorts and populations but are often particularly susceptible
29 to environmental changes. This raises concerns that future conditions, influenced by
30 anthropogenic changes such as ocean acidification and increasing oxygen minimum zones, will
31 greatly affect ecosystems by impacting developing larvae. Understanding the potential impacts
32 requires addressing present tolerances and the current conditions in which animals develop.
33 Here, we examined the changes in oxygen and pH adjacent to and within normally-developing
34 squid egg capsules, providing the first observations that the egg capsules, housing hundreds of
35 embryos, had extremely low internal pH (7.34) and oxygen concentrations ($1.9 \mu\text{mol L}^{-1}$). While
36 early-stage egg capsules had pH and oxygen levels significantly lower than the surrounding
37 seawater, late-stage capsules dropped dramatically to levels considered metabolically stressful
38 even for adults. The structure of squid egg capsules resulted in a closely packed unit of respiring
39 embryos, which likely contributed to the oxygen-poor and CO_2 -rich local environment. These
40 conditions rivaled the extremes found in the squids' natural environment, suggesting they may
41 already be near their metabolic limit and that these conditions may induce a hatching cue. While
42 squid may be adapted to these conditions, further climate change could place young, keystone
43 squid outside of their physiological limits.

44

45 Introduction

46
47 Shifts in oceanic chemistry, such as changes in available oxygen (O₂) and decreasing pH,
48 are of growing concern given their potential impacts on marine organisms and the ecosystems
49 they support (Pörtner et al. 2004, Seibel and Childress 2013, Rosa et al. 2014). Dissolved O₂ is
50 necessary for cellular respiration, but in many oceanic regions O₂ levels are declining and
51 oxygen minimum zones are expanding (Stramma et al. 2012). These changes are attributed to
52 several factors including lower sea-surface O₂ concentrations, local eutrophication events, and
53 reduced ventilation from ocean warming. Decreasing O₂ levels are compounded by increasing
54 carbon dioxide (CO₂) concentrations, largely from fossil fuel burning, which drive ocean
55 acidification (Caldeira & Wickett 2003). Despite concerns regarding future ocean conditions, the
56 baseline environmental conditions organisms currently face still requires substantial attention,
57 especially in highly dynamic coastal ecosystems where parameters such as O₂ and pH fluctuate
58 dramatically (Gobler et al. 2014, Wallace et al. 2014). Consequently, there are large uncertainties
59 when predicting the influence of current and future environmental changes on key marine taxa.
60 Baseline data affords a better understanding of these animals' current tolerances and contributes
61 to the reduction of these uncertainties.

62
63 In nearly all marine environments, young, developing animals appear particularly
64 susceptible to changing ocean conditions, with effects such as impaired development and
65 reduced size having been shown for a variety of species (Kurihara 2008, Ries et al. 2009, Rosa et
66 al. 2013). These impacts have been suggested to reduce recruitment success and, consequently,
67 could reduce population abundances (Munday et al. 2010). Marine invertebrates that deposit
68 calcareous skeletons have received much attention with their young showing vital changes (i.e.,
69 growth, structure) when raised under ocean acidification or hypoxic conditions (Hoegh-Guldberg
70 et al. 2007, Ries et al. 2009, Gobler et al. 2014). Impacts to soft-bodied invertebrates are
71 seemingly less understood, yet they too have calcified structures, and are often physiologically
72 limited by pH, aragonite concentrations, and O₂ levels (Radtke 1983, O'Dor et al. 1994, Pörtner
73 et al. 2004, Rosa et al. 2013). Any mechanism that may even slightly reduce the early-life
74 survival rates of marine organisms can have major repercussions on adult population sizes and,
75 in the case of keystone taxa, overall ecosystem health (Houde 1987, 2008).

76
77 Cephalopods, particularly squid, are an ecologically and economically key taxon,
78 providing a central trophic link in many marine food webs and 15-20% of global fisheries
79 landings and values (Boyle & Rodhouse 2005, Hunsicker et al. 2010). The Loliginid squid are
80 the primary commercial cephalopod of the western North Atlantic and support a fishery of
81 approximately 16,600 mt yr⁻¹ (NOAA 2010). Occasionally cited as keystone taxa, squid play a
82 central role in food webs as predator and prey to a wide array of taxa that occupy different
83 trophic levels (Clarke 1996). Cephalopods are no exception to the potential impacts of changing
84 ocean conditions. Increased pCO₂ can cause significant increases in development time, decreases
85 in hatchling size, and changes to statolith structure in squid (*D. pealeii*) (Kaplan et al. 2013). In
86 adults, decreased pH can impair the O₂ binding capacity of haemocyanin, the squid respiratory
87 protein (Pörtner 1990). Even in today's oceans, adult squid are considered to live near the edge
88 of O₂ limitation, particularly as they exercise (Pörtner et al. 1991, Seibel 2007, Seibel &
89 Childress 2013). Hence, lower metabolic rates occur across different squid taxa as respective
90 environmental O₂ concentrations decrease (Seibel 1997). Small decreases in ambient pH or O₂

91 are thought to endanger haemocyanin's ability to bind sufficient O₂ or otherwise limit O₂ uptake,
92 and would likely impair the squid's high energetic demand (Pörtner et al. 2004, Seibel &
93 Childress 2013).

94
95 Squid recruitment is largely driven by environmental factors (Dawe et al. 1990) and
96 environmental conditions play a large role in migrations, distribution, growth, and spawning
97 (Boyle & Rodhouse 2005, Zeidberg et al. 2011). Because cephalopod abundances are directly
98 tied to the success of early life history, growth, and survival (Boyle & Rodhouse 2005, Foote et
99 al. 2006), environmental changes such as ocean acidification or decreased O₂ availability could
100 directly impact populations. With growing evidence that ocean acidification will be amplified by
101 hypoxia and eutrophication in coastal waters, it is becoming increasingly important to consider
102 the interaction of these environmental parameters (Melzner et al. 2013, Cai et al. 2011, Wallace
103 et al. 2014), particularly on the susceptible early life stages. Adjacent, near-shore estuarine
104 habitats where squid such as *D. pealeii* are occasionally found may vary substantially in pH and
105 O₂, conditions that are exacerbated by eutrophication (Wallace et al. 2014, Baumann et al. 2015).
106 Yet, for *D. pealeii* and many other squid species there are few data addressing the epi-benthic,
107 coastal environment (pH, O₂, flow) where the majority of reproductive adults are harvested, and
108 thus, the conditions that most egg capsules naturally experience.

109
110 There is a growing body of literature that addresses O₂ availability or O₂ and pH
111 conditions associated with developing mollusks (Booth 1995, Cohen & Strathmann 1996, Moran
112 & Woods 2007). Work on cephalopods has largely focused on cuttlefish (Cronin & Seymour
113 2000, Gutowska & Melzner 2009, Dorey et al. 2013), a taxon in which a single embryo develops
114 in individual capsules. Adult Loliginids, like many coastal squid, lay their eggs in gelatinous
115 capsules on the benthos with each egg capsule densely housing 150-200 embryos (Hanlon &
116 Messenger 1996). These animals undergo rapid growth, becoming fully developed in 12-14 days
117 at 20° C (McMahon & Summers 1971), with the capsule expanding in size to accommodate this
118 growth (Hanlon et al. 1983). Although most hatching occurs during the night and certain
119 physical disturbances (such as handling) may induce hatching (Hanlon & Messenger 1998,
120 Zeidberg et al. 2011), the natural cues or catalysts for squid egg hatching (besides full
121 development) are not well established. While recent work has considered the respiration of squid
122 embryos, individuals were removed from their capsules for respirometry measurements creating
123 a design unlike that of nature, and potentially promoting premature stress and hatching (Rosa et
124 al. 2012, Rosa et al. 2014). Thus, to date, squid embryos consume an unknown amount of O₂ and
125 produce an unquantified amount of CO₂, all within a semi-permeable capsule, the structure of
126 which likely alters the exchange of O₂ and CO₂. Further, it remains poorly understood how a
127 population of fast growing, highly active cephalopod embryos within a capsule influences pH
128 and O₂ within the capsule or adjacent water, and how this influence may vary with development,
129 embryo size, and increases in O₂ demand and CO₂ respiration. Such studies would require a
130 detailed profile of the egg capsule and the surrounding physical boundary layer of intact capsules
131 as has been done with metabolically slower gastropods and polychaetes (Chafee & Strathmann
132 1984, Booth 1995, Cohen & Strathmann 1996, Moran & Woods 2007). For example, in many
133 marine gastropods which lay benthic egg clutches, intracapsular O₂ availability substantially
134 affects embryo development rates (Booth 1995, Strathmann & Strathmann 1995). Local
135 environmental conditions can also affect oxygen uptake and consequent embryo condition
136 (Cohen & Strathmann 1996, Cancino et al. 2011). Further, the physical boundary layer

137 surrounding egg capsules is a function of the physical characteristics of water flow rates and the
138 roughness of the capsule surface and may significantly alter exchange across organismal
139 boundary layers. The variation of this boundary layer due to fluctuating flow rates in coastal
140 ecosystems has not been considered in experiments of changing ocean conditions or metabolism
141 on cephalopod egg capsules.
142

143 To address these unknowns and provide a better understanding the natural pH and O₂
144 conditions associated with a densely populated cephalopod egg capsule, this work sought to
145 quantify (1) the O₂ and pH levels within egg capsules where embryos develop, (2) the egg
146 capsule O₂ consumption and pH change across embryonic development, and (3) the pH and O₂ in
147 the boundary layer adjacent to the capsule. These data were then placed in the context of current
148 data on thresholds and pH and O₂ limits for squid, to highlight what data are needed for this
149 critical developmental stage.
150

151 **Methods**

152

153 Experiments were conducted at the Woods Hole Oceanographic Institution (WHOI), MA
154 in August-September, 2014. The collection of adult squid was conducted under Massachusetts
155 Division of Marine Fisheries research permit #152087. Husbandry and animal care were
156 performed in accordance with guidelines as approved by WHOI's Institutional Animal Care and
157 Utilization Committee. Squid, *D. pealeii*, were trawl-caught in Vineyard Sound, MA, USA on
158 two occasions in 10-20 meters of water. Adults in healthy condition (free of cuts and scrapes)
159 were hand-selected from the group, gently placed in individual buckets, and were immediately
160 transported to a 500L holding tank at WHOI and maintained in 14°C cooled, filtered, flowing
161 local water. Within ~48-72h the squid bred, laying eggs capsules in a mass on the tank bottom.
162 Egg masses (~30 capsules mass⁻¹) were transferred to either a 38L aquarium in which water was
163 replaced daily, or a 100L flow-through aquarium, both of which were filled with local filtered-
164 seawater maintained at 20°C, which is the average temperature for Vineyard Sound during the
165 study period (19.4 ± 0.68 (± SD), data from Martha's Vineyard Coastal Observatory,
166 <http://www.whoi.edu/page.do?pid=70177>). Individual egg capsules were separated from the egg
167 mass immediately prior to profiling, attached on top of 5mm rigid plastic mesh using zip ties at
168 the leading edge of the capsule, and transferred to a 0.5L glass container or a custom 9.5L
169 recirculating micro-flume, both filled with the same filtered 20°C seawater. All measurements
170 and incubations were done under ambient laboratory light conditions. A micromanipulator
171 (Unisense, DK) was used to vertically profile up to and within the egg capsules (Fig 1).
172

173 A FireStingO₂ optical oxygen sensor (50µm sensing tip) and meter (Pyroscience, GE)
174 and liquid ion exchange (LIX) pH sensors (5-20µm sensing tip) were used to measure profiles.
175 LIX pH sensors were constructed and used following (Gieseke & de Beer 2004). To describe
176 briefly, glass capillaries were pulled to a tip diameter of 5-20 µm and the glass was silinized
177 using N,N-dimethyltrimethylsilylamine (Sigma, USA) in a sealed glass container at 200 °C to
178 make the glass hydrophobic. Poly-vinyl chloride stabilized H⁺ sensitive membranes (H⁺
179 ionophore II, Sigma, USA) were pulled into the capillary tips, and the electrode was back-filled
180 with a 300 mmol L⁻¹ potassium chloride, 7.0 pH, 50 mmol L⁻¹ phosphate buffer. The micro
181 electrodes were finished by sealing a silver chloride plated 0.25 mm diameter silver wire into the
182 back of the capillary. The electrochemical circuit was completed with a reference electrode

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

187
188 The pH sensors were calibrated using NIST-traceable buffers at $20\text{ }^{\circ}\text{C}$ and cross-checked
189 before and after each profile with a commercial pH sensor (Hach, USA). The Pyroscience O_2
190 optodes were calibrated using a saturated sodium ascorbate solution (0% O_2) and water-saturated
191 air according to manufacturer instructions. To ensure sensors were not damaged or otherwise
192 affected by profiling through the egg capsules, sensors were returned to the ambient water after
193 reaching the center of the egg capsules to confirm that the sensors showed consistent readings
194 with the beginning of the profile

195
196 The recirculating mini-flume consisted of a divided 9.5L aquaria connected by a passage
197 0.07 high, 0.07 wide, and 0.3 m long. Water was pumped between the aquaria halves using a
198 small pump and the flow adjusted using a ball-valve. Flow rates were evaluated with simple
199 discharge-area-time relationships. Sensors were located using the micromanipulator and a
200 forward-looking adjustable (0-25x) dissection scope (Zeiss, GE), all mounted to a sturdy
201 microprofiling base station (Unisense, DK). The mini-flume water was changed daily using the
202 filtered, local seawater at $20\text{ }^{\circ}\text{C}$. Static (no-flow) profiles were determined in 0.5L glass
203 containers within the same microprofiling base station, and filled with water from the aquaria the
204 capsules were incubated in. Egg capsules were incubated in the flume at a specific flow rate for
205 at least 1 hour prior to profiling.

206
207 A coarse vertical profile (1000 μm increments) was measured in the water column down
208 to the capsule “boundary layer”, or the fluid layer around the egg capsule where diffusive
209 transport is of primary importance, defined here by the location where large O_2 concentration
210 changes were observed (e.g. Figure 1, Gieseke & de Beer 2004). At the boundary layer
211 measurement increments were decreased to 100 μm to better resolve the concentration gradient.
212 The sensors were then pushed into the egg capsule and the profile continued until reaching the
213 egg capsule center, determined using a micrometer in the dissection scope, the egg capsule
214 diameter, and the visible location of the sensor tip. Care was taken to prevent the puncture of
215 individual embryos; the small sensor movements allowed the embryos to shift laterally, allowing
216 the sensors to remain within the intracapsular fluid. Each profile was done on a new egg capsule
217 in the range of 1-3 or 10-13 days-old. Differences between the ambient conditions and conditions
218 in the center of the different aged capsules were determined by one-way ANOVAs with
219 differences between these groups determined by Tukey post-test (Figure 2, Table 1). Due to the
220 seasonal cessation of squid breeding, testing of flow effects on egg capsule profiles could only be
221 conducted with unfertilized egg capsules. However, these provided initial baseline profiles of
222 egg capsule respiration due to microbial biomass (i.e., no metabolism of developing embryos)
223 and the effects of water flow past capsules at low ($0.01\text{ m}\cdot\text{s}^{-1}$) and high ($0.1\text{ m}\cdot\text{s}^{-1}$) current
224 velocities utilizing the recirculating micro-flume.

225 226 **Results**

227 Newly-laid egg capsules demonstrated significantly lower O_2 concentrations and pH
228 relative to the ambient water, with O_2 dropping from $\sim 200\text{ }\mu\text{mol L}^{-1}$ O_2 to 160 at the capsule

229 center and a pH decrease from 8.0 to 7.8 (Figure 1A, C). This difference increased substantially
230 with egg development (Figure 1B, C). After 10-13 days of development the egg capsule centers
231 contained only trace amounts of O₂ (1.9 ±1.1 μmol L⁻¹) and had a pH of 7.34 ±0.01 (±SD).

232
233 The steep gradients between the ambient water and the egg capsule allowed for the
234 calculation of diffusive O₂ flux across the boundary layer around the egg capsule (Figure 1). The
235 dominant transport process in this boundary layer is diffusion; therefore Fick's Law of Diffusion
236 can be used to determine exchange across the egg capsule surface. The flux = $\partial O_2 / \partial x * D$, where
237 D is the diffusion coefficient of O₂, and x is the depth (Gieseke & de Beer 2004). The depth of
238 the boundary layer (x) was determined from the concentration profiles and the O₂ gradient was
239 determined from the O₂ gradient between the ambient water and the egg capsule surface. The
240 fluxes revealed a 10-fold increase in egg capsule O₂ consumption over a 10-day period (0.060 to
241 0.595 μmol cm⁻² min⁻¹ for the 1-3 and 10-13 day-old capsules, respectively). Applying Fick's
242 Law of Diffusion to the capsule boundary layer allows for the determination of the time point
243 when the maximum physical transport into the capsule is exceeded by the capsule metabolic
244 requirement (indicating significant hypoxic stress). For example, using the measured boundary
245 layer thickness, the maximum possible O₂ gradient (~200 μmol L⁻¹ mm⁻¹), and assuming a linear
246 increase in O₂ consumption with capsule age, the time when the maximum physical transport of
247 O₂ is exceeded by egg O₂ consumption (0.84 μmol cm⁻² min⁻¹) was 15.8 days.

248
249 The unfertilized egg capsules had similar O₂ concentration changes and profiles to the 1-
250 3 day-old egg capsules (Figure 2; Table 1). The former is primarily due to the metabolism of
251 capsule-associated microbial communities (Barbieri et al. 2001) and suggests a relatively small
252 measureable metabolic contribution by the 1-3 day-old embryos. The calculated O₂ fluxes were
253 0.073, 0.088, and 0.098 μmol cm⁻² min⁻¹ for unfertilized egg capsules under no-flow, low-flow,
254 and high-flow conditions, respectively. The thickness of the boundary layer decreased with flow
255 (2.0, 0.7, and 0.4mm for the no-flow, low-flow, and high-flow conditions, respectively).

256
257 All of the egg capsules used in this study hatched viable squid paralarvae, with the
258 exception of the unfertilized capsules (characterized by no change in size or visible growth).
259 Hatching success was not evaluated as a part of this work. The developing egg capsules visibly
260 increased in volume as the embryos grew (Table 1), leading to the deeper profiles in the 10-13
261 day-old egg capsules.

262 263 **Discussion**

264
265 Conditions in the full-term embryo capsules were unexpectedly low in both O₂ and pH,
266 reaching levels that are often considered adverse to many pelagic taxa (Stramma et al. 2012).
267 These O₂ levels were below that of water adjacent to the capsules and that of the local
268 environment where these capsules are often found, as well as even that of Atlantic oxygen
269 minimum zones (Karstensen et al. 2008). Although some oceanic deep-sea squid species have
270 shown tolerances and even affinities to low O₂ levels (Gilly et al. 2012, Seibel 2013), active,
271 coastal, adult Loliginid squids are typically considered near the edge of metabolic O₂ capabilities
272 and somewhat intolerant of the conditions measured here (Pörtner 2002). The decrease of O₂
273 levels by 99% to near-anoxic conditions across growth is larger than the hypoxic conditions
274 observed for similar cephalopod species that have a single egg per capsule (75% decrease (Dorey

275 et al. 2013), 86% decrease (Cronin and Seymour 2000), 62% decrease (Gutowska and Melzner
276 2009), 85% decrease (Rosa et al. 2013)) suggesting the densely packed egg capsule structure
277 leads to a an extremely high O₂ demand, as has been found in a number of gastropod and
278 polychaete species housing multiple eggs per capsule (Chafee & Strathmann 1984, Booth 1995,
279 Cohen & Strahmann 1996, Moran & Woods 2007). The observation of hatching and healthy
280 paralarvae was surprising based on previous results from studies on cephalopods, and suggests
281 these conditions may not have induced extreme stress, similar to results found in other multiple-
282 egg per capsule species.

283
284 The boundary effects of the capsule suggest that encapsulation of the many embryos
285 likely contributes to the lower O₂ and pH (Chafee & Strathmann 1984, Booth 1995, Moran &
286 Woods 2007), resulting in conditions that are substantially lower than observed for single-egg-
287 per-capsule species (e.g., cuttlefish (Rosa et al. 2013)). The encapsulation of embryos has been
288 proposed as a mechanism to protect embryos against ocean acidification through the buffering
289 capacity of intracapsular fluids (Ellis et al. 2009, Fernandes & Podolsky 2012) but our data
290 suggest that encapsulation causes reduced pH conditions around embryos. We expect even
291 lower pH and O₂ values inside the egg capsules of squid raised in elevated ocean acidification or
292 low oxygen conditions (as seen in some taxa (Rosa et al. 2013, Noisette et al. 2014)). However,
293 it is not clear whether these lower pH or O₂ conditions would lead to greater impacts or perhaps
294 support adaptation to future, changing conditions.

295
296 Squid, particularly muscular, shallower species such as the taxa studied here, are
297 considered relatively intolerant to small changes in pH (Pörtner et al. 2004). The blood pH for
298 these adult squid is typically near 7.6 (Pörtner 1990) with some exceptions for specialized
299 species living in OMZ or the deep ocean (Seibel 2013, Seibel and Childress 2013). The
300 intracapsular levels of pH 7.34 noted here were unexpected for such energetic coastal squid and
301 were also well below environmental levels which have induced developmental changes in young
302 squid (Kaplan et al. 2013, Rosa et al. 2014). This implies that, during prior studies, pH values
303 inside the experimental capsules (Kaplan et al. 2013, Rosa et al. 2014) were even lower, perhaps
304 suggesting that despite these extreme conditions, young squid may be more tolerant than
305 previously considered. Further, the measured levels of this study were near the limit of predicted
306 pH-dependent blood pigment O₂ affinity (Pörtner 1990); at a lower pH, blood might not
307 effectively take up O₂. These embryos may already be at relatively inefficient metabolic levels
308 due to the combined effect of low pH and O₂ or their haemocyanin pigment may have an
309 improved affinity compared to adults, for example, due to the presence of different isoforms of
310 haemocyanin in cephalopod embryos that may be more efficient at O₂ binding (Thonig et al.
311 2014). However, the higher surface area-to-volume ratio and importance of cutaneous O₂ uptake
312 in squid embryos indicates that pigment-mediated O₂ exchange would likely be less important.
313 While pH is a likely stressor for many squid, the low O₂ concentrations may dominate in limiting
314 metabolism and energetics (Seibel et al. 1997, Seibel & Childress 2013). Thus, the low O₂ levels
315 of egg capsules seen here reinforce concerns of expanding OMZs and deoxygenation of ocean
316 waters, particularly if the limits of organismal adaptation are reached.

317
318 Certainly these squid may be adaptable to changes, as seen with tolerance to low O₂
319 conditions in the young of some more specialized squid species (Seibel 2013, Trübenbach et al.
320 2013, Seibel et al. 1997). While the conditions shown here are relatively extreme for the open

321 ocean, they may be less stressful for coastal and estuarine organisms, which may have a greater
322 range of tolerances (Murray et al. 2014). Based on data from adult squid and multiple other taxa
323 (Pörtner et al. 2004, Seibel and Childress 2013), these squid may already be near a physiological
324 limit, or less adaptable in the face of future ocean biogeochemical changes. Neither the optimal
325 nor threshold O₂ and pH levels for embryonic development have been defined, so we do not
326 know whether these conditions place substantial stress on the developing squid. The effect of the
327 low intracapsular pH and O₂ observed here requires further study to determine how squid may be
328 affected by future O₂ and CO₂ conditions. Understanding the mechanisms and the potential for
329 developing squid embryos to withstand these conditions will inform our expectations for how
330 these and other organisms may cope with projected global ocean changes.

331
332 Both pH and O₂ levels decreased over time, reflecting the increased size and energetic
333 demand of developing squid. As shown elsewhere (Pörtner et al. 2004, Seibel and Childress
334 2013), there are limits to squid pH and oxygen tolerances; we suggest here that these levels may
335 even act as an embryonic hatching cue. The maximum time to hatching was estimated from the
336 maximum physical O₂ gradient across the egg capsule boundary layer (Figure 2D). This
337 maximum exchange rate under no-flow conditions suggests a maximum hatching time of 15.8
338 days under the observed conditions, which is consistent with previously observed hatching times
339 of 12-14 days (Kaplan et al. 2013). The decrease to such low levels within the capsule may act as
340 an embryonic hatching cue, and compounding O₂ and pH changes in the surrounding
341 environment may induce premature hatching. Water flow over the capsule likely plays a role as
342 the capsule boundary layer thickness decreased with increasing flow (Figure 2C) and O₂
343 exchange increased in the unfertilized egg capsules suggesting enhanced exchange across the
344 capsule surface due to current flow. Previously, hatching has been observed at night and
345 explained as a mechanism to reduce being eaten by visual predators (Zeidberg et al. 2011).
346 Conversely, hatching may be induced during hydrodynamically calm periods during the night
347 when O₂ is reduced by ecosystem respiration and the absence of photosynthesis, for example,
348 during a nighttime slack tide. The enhanced exchange with flow also suggests that egg laying
349 locations that have active hydrodynamics may lead to faster embryonic development and reduced
350 stress by low pH and O₂ conditions (Chafee & Strathmann 1984, Cohen & Strathmann 1996,
351 Zeidberg et al. 2011). Therefore, ocean acidification and hypoxia experiments should consider
352 the effects of flow-enhanced exchange between organisms and the ambient seawater.

353

354

355 **References**

356

357 Barbieri E, Paster B, Hughes D, Zurek L, Moser D, Teske A, Sogin M (2001) Phylogenetic
358 characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules
359 of the squid *Loligo pealei* (*Cephalopoda:Loliginidae*). *Environmental Microbiology*
360 3:151-167

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

363 Baumann H, Wallace RB, Tagliaferri T, Gobler CJ (2015) Large natural pH, CO₂ and O₂
364 fluctuations in a temperate tidal salt marsh on diel, seasonal, and interannual time
365 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 Biol 198: 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 Bull 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

456 Pörtner H-O (1990) An analysis of the effects of pH on oxygen binding by squid (*Illex*
 457 *illecebrosus*, *Loligo pealei*) haemocyanin. J Exp Biol 150:407-424
 458 Pörtner H, Langenbuch M, Reipschläger A (2004) Biological Impact of Elevated Ocean CO₂
 459 Concentrations: Lessons from Animal Physiology and Earth History. J Ocean 60
 460 Pörtner HO (2002) Environmental and functional limits to muscular exercise and body size in
 461 marine invertebrate athletes. Comp Biochem Physiol A 133:303-321
 462 Pörtner HO, Webber DM, Boutilier RG, O'Dor RK (1991) Acid-base regulation in exercising
 463 squid (*Illex illecebrosus*, *Loligo pealei*). American Journal of Physiology 261:R239-R246
 464 Radtke RL (1983) Chemical and structural characteristics of statoliths from the short-finned
 465 squid *Illex illecebrosus*. Marine Biology 76:47-54
 466 Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO₂-
 467 induced ocean acidification. Geology 37:1131-1134
 468 Rosa R, Pimentel MS, Boavida-Portugal J, Teixeira T, Trübenbach K, Diniz M (2012) Ocean
 469 warming enhances malformations, premature hatching, metabolic suppression and
 470 oxidative stress in the early life stages of a keystone squid. PLoS One 7:e38282
 471 Rosa R, Trübenbach K, Pimentel MS, Boavida-Portugal J, Faleiro F, Baptista M, Dionísio G,
 472 Calado R, Pörtner HO, Repolho T (2014) Differential impacts of ocean acidification and
 473 warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). J Exp Biol
 474 217:518-525
 475 Rosa R, Trübenbach K, Repolho T, Pimentel M, Faleiro F, Boavida-Portugal J, Baptista M,
 476 Lopes VM, Dionísio G, Leal MC (2013) Lower hypoxia thresholds of cuttlefish early life
 477 stages living in a warm acidified ocean. Proceedings of the Royal Society B: Biological
 478 Sciences 280:20131695
 479 Seibel BA (2007) On the depth and scale of metabolic rate variation: Scaling of oxygen
 480 consumption rates and enzymatic activity in the class cephalopoda (mollusca). J Exp Biol
 481 210:1-11
 482 Seibel BA (2013) The jumbo squid, *Dosidicus gigas* (Ommastrephidae), living in oxygen
 483 minimum zones II: Blood–oxygen binding. Deep Sea Research Part II: Topical Studies in
 484 Oceanography 95: 139-144
 485 Seibel BA, Childress JJ (2013) The real limits to marine life: a further critique of the Respiration
 486 Index. Biogeosciences 10: 2815-2819
 487 Seibel BA, Thuesen EV, Childress JJ, Gorodezky LA (1997) Decline in pelagic cephalopod
 488 metabolism with habitat depth reflects differences in locomotory efficiency. The
 489 Biological Bulletin 192:262-278
 490 Stramma L, Prince ED, Schmidtko S, Luo J, Hoolihan JP, Visbeck M, R.Wallace DW, Brandt P,
 491 Körtzinger A (2012) Expansion of oxygen minimum zones may reduce available habitat
 492 for tropical pelagic fishes. Nat Clim Chg 2:33-37
 493 Strathmann RR, Strathmann MF (1995) Oxygen supply and limits on aggregation of embryos.
 494 Journal of the Marine Biological Association of the United Kingdom 75:413-428
 495 Thonig A, Oellermann M, Loeb B, Mark FC (2014) A new haemocyanin in cuttlefish (*Sepia*
 496 *officinalis*) eggs: sequence analysis and relevance during ontogeny. EvoDevo 5:1-13
 497 Trübenbach K, Pegado MR, Seibel BA, Rosa R (2013) Ventilation rates and activity levels of
 498 juvenile jumbo squid under metabolic suppression in the oxygen minimum zone. J Exp
 499 Biol 216:359-368
 500 Wallace RB, Baumann H, Gear JS, Aller RC, Gobler CJ (2014) Coastal ocean acidification: The
 501 other eutrophication problem. Estuarine, Coastal and Shelf Science 148:1-13

502 Zeidberg LD, Isaac G, Widmer CL, Neumeister H, Gilly WF (2011) Egg capsule hatch rate and
503 incubation duration of the California market squid, *Doryteuthis* (= *Loligo*) *opalescens*:
504 insights from laboratory manipulations. *Marine Ecology* 32:468-479
505

506 **Acknowledgments**

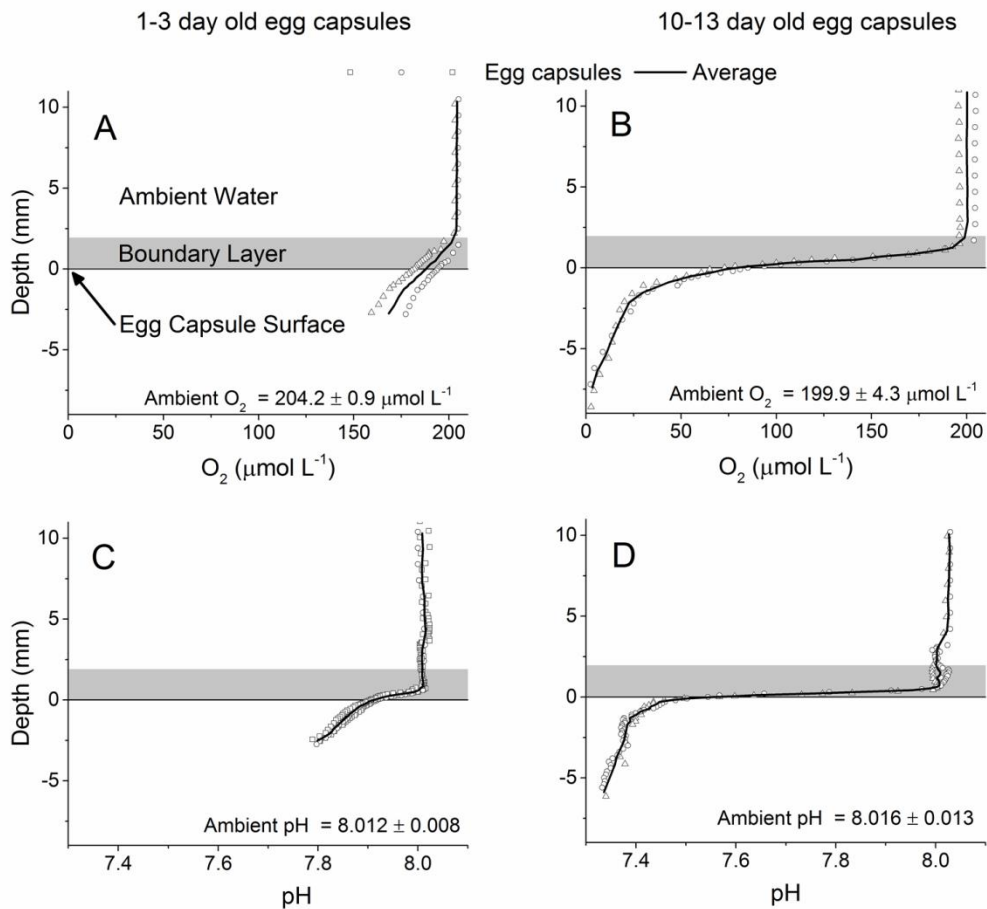
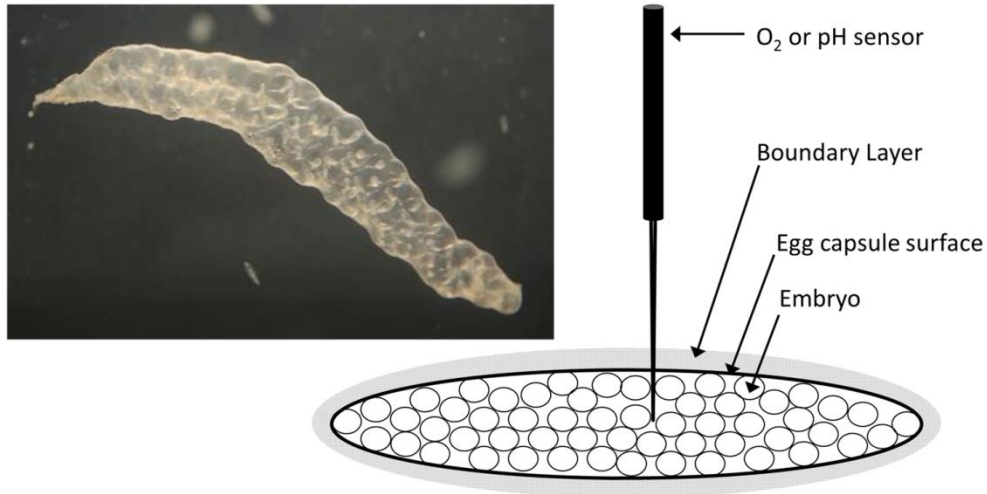
507
508 The authors thank the MBL-MRC team for squid collections, and the advice and support from R.
509 Hanlon, S. Gallagher, R. Galat, D. McCorkle and M. Charette. This work was supported by a
510 NSF Ocean Acidification grant (#1220034; TAM) and the WHOI Ocean Climate Change
511 Institute (Ocean Acidification Initiative; MHL). This manuscript was substantially improved by
512 comments from three anonymous reviewers.
513

514
515

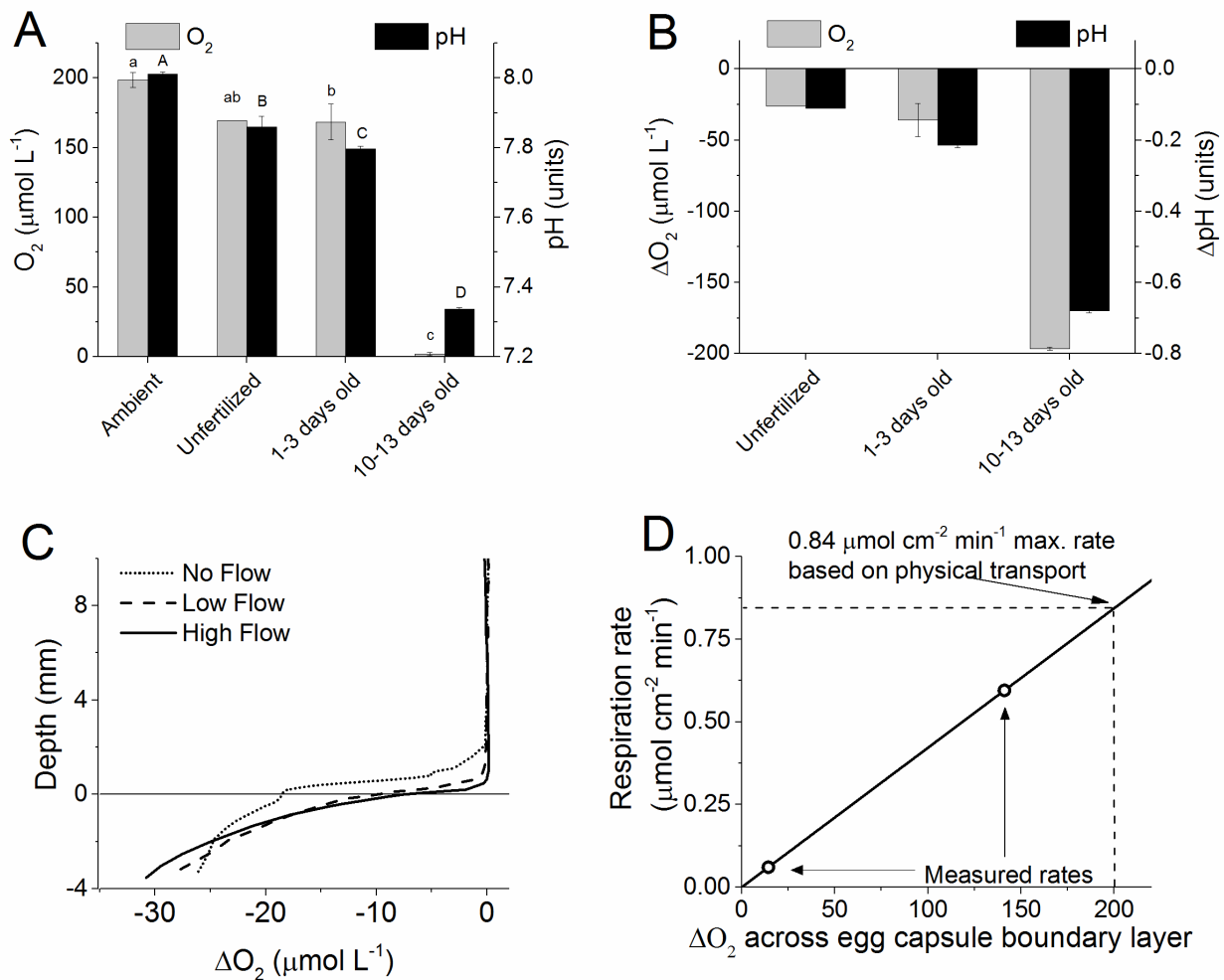
Table 1. Oxygen and pH in the ambient water and center of egg capsules

	Egg Capsule Diameter	Oxygen	pH
	(mm)	($\mu\text{mol L}^{-1}$)	(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.



518
 519 Figure 1. Egg capsule picture and schematic of profiling, boundary layer (grey shading), and egg
 520 capsule (top). Profiles of oxygen (A,B) and pH (C,D) in 1-3 day-old (left) and 10-13 day-old
 521 (right) egg capsules. Shapes indicate individual profiles in different egg capsules and the solid
 522 lines are the average profile. Photo credit: C. Zakroff.



523
 524 Figure 2. Oxygen concentrations and pH (A) in ambient water and the center of different aged
 525 egg capsules showing significant variation in both oxygen and pH (Table 1); letters indicate
 526 differences between groups (using Tukey post-tests). (B) The change in oxygen and pH in the
 527 center of egg capsules relative to the ambient water conditions. (C) The effect of different flow
 528 rates on oxygen profiles in unfertilized egg capsules and the compression of the boundary layer
 529 where each line represents the average of three profiles. (D) The maximum respiration rate under
 530 no-flow conditions (see text) indicating egg capsules ~16 days-old will be significantly stressed
 531 during hydrodynamically calm periods.
 532