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Consequences of environmental variability for spawning and embryo development of
inshore market squid *Doryteuthis opalescens*

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Oceanography

by

Michael O. Navarro

Committee in charge:

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2014

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The Dissertation of Michael O. Navarro is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014

DEDICATION

For my grandfather, Michael E. Navarro.

EPIGRAPH

The true biologist deals with life, with teeming boisterous life,
and learns something from it,
learns that the first rule of life is living.

Ed Ricketts
“The Log of the Sea of Cortez”

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Consequences of environmental variability for spawning and embryo development of inshore market squid *Doryteuthis opalescens*

by

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Doryteuthis opalescens aggregate and migrate to the continental shelf during the last stage of their life presumably to select spawning sites that optimize the survival and fitness of their offspring. This dissertation examined the timing and site selection of spawning aggregations, and essential habitat requirements for their embryos. Squid embryos occurred every year for five years, across all seasons during most years (Winter 2009-Summer 2013) and were commonly observed to occur on coarse sands and on

submarine canyon walls. Essential embryo habitat was defined by observed depth (10-100 m depth), embryo density ($0.1-350.1 \text{ capsules}\cdot\text{m}^{-2}$), area ($0.15-7.32\cdot 10^6 \text{ m}^2$), by repeated use, and by association with high $[\text{O}_2]$ and pH (and low $p\text{CO}_2$). ROV observations of embryos recorded $[\text{O}_2]$ from 70-280 μM , pH from 7.65-8.10, T from 9.8-18.1 $^\circ\text{C}$, and S from 33.3-33.9 PSU and that $[\text{O}_2]$, pH and T were higher (and S lower) in areas where embryos were present. Embryos exposed to combined low pH and low $[\text{O}_2]$ in the laboratory had a 16.7 % longer development time, remained at earlier development stages more often and had 54.7% smaller statolith area. Embryo dorsal mantle length and statoliths were bigger and yolk reserves were smaller in a low pH only laboratory treatment relative to those in a low $[\text{O}_2]$ only treatment. Geochemical testing for pH and $[\text{O}_2]$ effects evaluated element ratios (B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, Pb:Ca, U:Ca) of embryonic statoliths. Uranium:Ca was eight-times higher in statoliths exposed to low pH and low $[\text{O}_2]$ than those exposed to high pH and $[\text{O}_2]$.

This thesis provides evidence that squid spawn and hatch continuously on the SCB, that adults select sites that are exposed to relatively higher $[\text{O}_2]$ and pH, embryos develop poorly when exposed to low $[\text{O}_2]$ and pH, and that embryo statolith geochemistry can reflect sublethal levels of $[\text{O}_2]$ and pH. Several options are available for inclusion of climate change into adaptive management policy including mapping and monitoring embryo habitat over the range of *D. opalescens* and development of research initiatives inclusive of commercial fishers.

CHAPTER 1.

Introduction

Many exploited-marine species are declining in abundance as a result of climate change across the globe (Pauly 2010, Brander 2010). Pauly (2010) suggests the global decline to be 20-30% by 2050. Theoretical evidence from Pauly (2010) supports the idea that changes in dissolved oxygen and carbon dioxide concentration occurring within each of the world's oceans will lead to change in the distribution (via horizontal and vertical migration) and abundance of organisms based on species-specific physiology. Globally, empirical evidence shows that many historically dominant fisheries are declining and that commercial exploitation of species is now shifting to new clades. For example, global marine groundfish harvests are decreasing while those of cephalopods are increasing (Caddy and Rodhouse 1998). Although some fishery oceanographers predict fishers will have to fish down the food web (Pauly and Palomares 2005) the increased fishing of squid may be more of an example of a lateral shift in the fishing.

Cephalopods may be an important exception to this general rule of decline of abundance for exploited species. First, the overall biomass of cephalopods has been estimated to be at least equal to that of fish (Clarke 1987). Second, some cephalopods including many species of squid can act as ecological equivalents of fish in terms of their trophic position (Pauly 1998). Therefore, it is a reasonable hypothesis to suggest that disproportionate extraction of fish biomass may "release" certain species of cephalopods and that the global biomass of cephalopods may actually be increasing (Pecl and Jackson 2008). Regardless, global fisheries are increasingly extracting cephalopods, especially

squid. Prior to 1969, Japan was the primary country capturing cephalopods (Sonu 1993). Since then, global cephalopod fisheries yearly totals have grown six-fold, from around 700,000 tonnes in 1969 to a high of ~4.3 million tonnes in 2007 and most recently more than 4 million tonnes in 2012 (Bianchi et al. 2014).

Climate Change in the California Current Ecosystem

Many global fisheries are captured within the productive waters of eastern-boundary currents. Understanding how climate change is affecting eastern-boundary currents is fundamental to understanding the response of fishery species. Perhaps the most well studied of all the eastern-boundary currents is the California Current, making it an ideal location for the study of squid response to environmental change. The California Current Ecosystem (CCE) can be defined as a “system composed of physical-chemical-biological processes active within a space-time unit” (Lindeman 1942; but see Tansley 1935) where the space-time unit is the California Current System (CCS; Hickey 1979, Checkley and Barth 2009). The CCS can be divided into three major areas: northern, central, southern (Checkley and Barth 2009). Further, the anatomy of the CCS can be separated into a composite of several currents: California Current, Coastal Jet, Davidson Current, California Undercurrent (reviewed by Checkley and Barth 2009).

Climate change impacts specific to the California Current Ecosystem (CCE) include a warming of 1 °C over the last 100 years (Checkley and Barth 2009), shoaling of the upper-layer of the Oxygen Minimum Zone (Bograd et al. 2008, McClatchie et al. 2010, Gilly et al. 2013, Booth et al. 2014) and of the corrosive waters of the Carbon Maximum Zone (Feely et al. 2008). Die-offs stimulated by the shoaling of near-hypoxic

and hypercapnic waters have been observed at numerous coastal sites within the California Current Ecosystem (e.g. Oregon, USA, Grantham et al. 2004; Baja California Norte, Hernandez-Ayon et al. pers. comm.; King's Harbor, California, USA, Caron et al. pers. comm.). Low $[O_2]$ waters can become hypoxic when combined with increased oxygen demand from microbial respiration of the production stimulated by nutrient inputs (Nam et al. 2011, Connolly et al. 2010).

Hydrographic observations in the Southern California Bight, suggest that low-oxygen events occur at a variety of temporal scales, most prominently within the Spring (upwelling) season (Nam et al. 2011, Frieder et al. 2012, Send and Nam 2012, Booth et al. 2014). Continuous monitoring revealed that the oxygen and pH co-vary together most dominantly at the semidiurnal scales consistent with the K1 and M2 tidal cycles (Frieder et al. 2012). Low oxygen and pH are often connected through biological processes (i.e. respiration; Pauly 2010, Frieder et al. 2012). In this same area, current dynamics are also driven by the K1 and M2 cycle with a larger velocity near Point La Jolla (Parnell et al. 2006). The La Jolla submarine canyon is adjacent to the point, and submarine canyons are geological features that that can act to magnify upwelling (Allen and Madron 2009, Gilly et al. pers. comm) as well as downward cascading (Kampf 2005). The CCS is also highly impacted by ENSO cycles. The thermocline changes in depth depending on ENSO state and is most shallow during La Niña. In contrast, the thermocline is deepest during El Niño and intermediate during neutral years. Seawater below the thermocline has lower $[O_2]$ and pH in comparison to above (Nam et al. 2011). Changing physico-chemical exposure, such as expected to occur with climate change, can impact some

marine life by compressing (or expanding) their available habitat (McClatchie et al. 2010).

Commercial Fisheries in California, USA

Along with environmental changes within the CCE, the emphasis of commercial fisheries has shifted as well, especially in California, USA. The change in global fisheries, more specifically the rising global demand for squid, has increased demand for squid in the CCE (Vojkovich 1998). Prior to the start for the global demand for market squid (1863-1959), market squid constituted a minor commercial fishery in California (Vojkovich 1998, Brady 2008). Then in the 1960s, market squid became a major commercial fishery (Vojkovich 1998) continuing to grow until, in the 1990s, it became the most dominant fishery in terms of volume and value in California (Protasio 2013). Regionally, over 90% of the *D. opalescens* harvest occurs in the Southern California Bight (Protasio 2013). Over the last four years, this squid fishery netted over 60 million USD in ex-vessel value and, in 2012, 87.4% of the harvest was exported grossing an additional 121 million USD (Protasio 2013). Squid are exported to 45 countries but 72% of all exports are sold to China (Protasio 2013). In contrast, traditional Actinopterygian (bony fish) commercial fisheries in California have either stabilized or declined (www.calcofi.org). The rise of invertebrate fisheries in California is consistent with global trends. Last year, *Doryteuthis opalescens* take was 60% of the biomass for all species captured by the entire California commercial fishery fleet (Porzio 2013). A fishery has not been this dominant in California since the iconic sardine fishery of the 1930s.

***Doryteuthis opalescens* as a Model Species**

Doryteuthis opalescens, ranges from British Columbia, Canada continuously through to Baja California, Mexico (Okutani and McGowan 1969, Jereb et al. 2010). Adults migrate from feeding grounds centered in the mesopelagic and upper slope (Miller et al. 2008) to spawn on the seafloor of the continental shelf (Jereb et al. 2010, Zeidberg et al. 2011a). These migrating adults and their reproductive output are an important ecological link, transferring energy from the slope to the epipelagic waters above and on the seafloor of the continental shelf of the California Current System (CCS). They are also a forage species, providing food for many federally protected species of fish (Morejohn 1978) and tetrapods (Henkel and Harvey 2008). In a recent study of squid embryos in central and northern California, USA most capsules were observed at depths ranging from 20-70 m, with the highest levels of abundance in the Southern California Bight (SCB; Zeidberg et al. 2011a). On the shelf, squid chemically adhere embryo capsules onto a variety of substrates (sandy plains, rocky habitat, kelp, artificial structures, submarine canyon walls) where they develop on or near the seafloor (Navarro et al. 2013, Chapter 2).

Squid respond rapidly to environmental variability both in theory (Pauly 2010) and empirically (Jackson and Domeier 2003, Zeidberg et al 2004, Ceiola and Jackson, 2010) making them an ideal model to investigate fishery response to climate change. The population dynamics of *D. opalescens* most strongly respond to ENSO and squid abundance changes depending on the ENSO state (Reiss et al. 2004, Koslow and Allen 2011). The California market squid fishery captured the most squid, 117,890 mt, during La Niña (2000 and 2010; Kong 2001, Protasio 2013), and least squid, 2,457 mt, during El

Niño (1999; Yaremko 2000). The range of individual squid captured is between $3.1 * 10^9$ (maximum; 2000) to $3.3 * 10^7$ (minimum; 1999) squid (weight was converted to individuals using an estimation of 6-12 squid per pound; <http://www.jaemarseafood.com>). If fishery data mirror the squid population (Dorval et al. 2013), then population of market squid changes by two orders of magnitude depending on the environmental state of ENSO with the minimum abundance of squid numbering at least in the millions. Quantitative biomass estimates for the *D. opalescens* population in California have been made recently using fishery data (Dorval et al. 2013). Methods for quantifying the entire stock have been proposed (Cailliet and Vaughan 1983, Lipinski and Soule 2007).

The mechanisms that cause this close connection of squid to the environment have yet to be clearly identified or experimentally tested. El Niño is considered to be unfavorable for *D. opalescens*. During El Niño food availability may be limited for paralarvae (Zeidberg and Hamner 2002) and squid size is reduced relative to La Niña (Reiss et al. 2004). Jackson and Domeier (2003) used microstructure techniques on statoliths to determine age and found that large and young squid (e.g. 4 months old) spawned commonly during La Niña conditions in contrast to old (e.g. 9 months old) and small squid, which spawned during El Niño conditions. Spawning aggregations of squid and their embryos have been identified as the most tractable portion of the market squid life cycle (Cailliet and Vaughan 1983, Lipinski and Soule 2007) and site-attached benthic embryos are most susceptible to environmental change. The *D. opalescens* embryo stage is susceptible to environmental change because encapsulated embryos 1) are attached to the seafloor and unable to escape exposure to intruding low oxygen and low pH waters

and 2) each embryo has a fixed energy reserve (i.e. yolk) for embryogenesis and regulation of internal pH. Loliginid embryos are dependent on aerobic metabolism and can be negatively affected by low [O₂] (Roberts 2005, Zeidberg et al. 2011b). Prior to this dissertation, the documented *D. opalescens* embryonic stage response to climate change was largely limited to temperature.

As true for all loliginids, *D. opalescens* growth and life span is inversely related to temperature within a species-specific thermal tolerance envelope (Jackson and Domeier 2003, Ceiola and Jackson, 2010). Depending on environmental conditions these semelparous squid spawn at age 4-9 months but on average spawn at 6 months of age (Jackson and Domeier 2003). Four main life stages have been described for *D. opalescens* (Fields 1965): embryos within benthic egg capsules, paralarvae, juvenile, and adult. Embryogenesis within capsules lasts for four to eight weeks depending on temperature (Zeidberg et al 2011b).

Climate change (i.e. OMZ upper boundary shoaling, ocean acidification, warming) and increased fishing pressure (i.e. global fishery changes) are likely to affect California's largest commercial fishery species, the market squid *Doryteuthis opalescens*. To examine the effects of ocean deoxygenation and acidification on squid spawning and embryogenesis, this dissertation is composed of four parts.

Thesis Prologue

Prior to this work, very little was known about the environment that *D. opalescens* embryos experiences in nature. Chapter 2 characterized the critical habitat of embryonic squid *Doryteuthis opalescens*. It describes where and when squid embryos

occur as well as the density patterns of squid embryo beds. Further, this chapter describes the associated environmental factors that squid embryos are exposed to, with a focus on $[O_2]$ and pH levels. To do this, novel techniques were used to identify squid embryo habitat including high-frequency ROV sampling on the continental shelf, surveying the upper slope using Scripps ROV Triton (first dive for biology), and characterizing environmental factors (e.g. $[O_2]$ and pH) on the seafloor utilizing the recently developed seapHOx and its durafet sensor for continuous stable pH measurement (Martz et al. 2010). This chapter provides the first description of the environmental envelope for *D. opalescens* embryos (i.e. temperature, salinity, density, $[O_2]$, pH, pCO_2 , $\Omega_{\text{aragonite}}$) in the Southern California Bight.

In Chapter 3, laboratory experiments were performed to test whether low environmental $[O_2]$ and pH levels affect embryonic-squid development in a biologically significant manner. Two experiments were performed. In Experiment 1, embryo capsules were exposed to a treatment of high pH and $[O_2]$ or a treatment of low pH and $[O_2]$. To tease apart the magnitude of single stressor effects on squid-embryo development, a second experiment exposed encapsulated embryos to low pH only or low $[O_2]$ only. These tests provide insight into how ocean deoxygenation and acidification affect embryogenesis in terms of hatch timing and fitness.

In a companion analysis of the laboratory experiments used in Chapter 3, subsamples were used investigated in Chapter 4 to test whether environmental $[O_2]$ and pH levels affect the elemental composition (B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, Pb:Ca, U:Ca) of embryonic statoliths, biogenic carbonate structures that function as part of the gravity sensor in the squid. This is the first time squid statolith geochemistry has been explored

as a potential “recorder” of environmental [O₂] and pH. This chapter examines how these “signals” are generated and the possible utility of statolith geochemistry for future study in paleobiology and ecology.

Chapter 5 provides dissertation conclusions and contextualizes the findings of the dissertation using global perspective. Inshore squid face a different mix of environmental changes specific to the region of the world that they inhabit. For example, in some parts of the world, warming is the dominant factor driving squid population dynamics whereas in others deoxygenation is more important. This chapter discusses successful adaptive management strategies used for selected fisheries in the face of climate change. These strategies are then summarized and integrated to provide recommendations for the California market squid fishery adaptive management strategy for ocean deoxygenation and acidification.

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CHAPTER 2.

Essential Embryo Habitat of *Doryteuthis opalescens* in the Southern California Bight

Abstract

Spawning aggregations of the inshore market squid, *Doryteuthis opalescens*, are important because this forage species is a key link in the continental shelf ecosystem of the California Current System. Microcohorts of spawning *D. opalescens* were observed to use the continental shelf across all seasons during most years (from Winter 2009-Summer 2013) as evidenced by observations from SCUBA, a tow camera and remotely-operated vehicle (ROV) surveys of squid-embryo capsules on the seafloor. From the upper slope (100-400 m depth) to the shelf (10 - 100 m depth) essential embryo habitat was defined by embryo-bed depth, embryo capsule density, area, repeated use, and by association with [O₂] and pH (*p*CO₂). The [O₂], pH, temperature, and salinity conditions at *D. opalescens* embryo beds reflect a variable environment ([O₂]: 70-280 μmol kg⁻¹; pH: 7.65-8.10, T: 9.8-18.1 °C; S: 33.3 - 33.6 PSU). Based on ROV surveys, embryo capsule density was highest at the upper regions of the shelf (≤ 42 m), where temperature, [O₂] and pH are highest. Integrated mapping of embryo beds and environmental data (O₂ and T) within essential embryo-bed habitat across a broad spatial scale is needed to make informed policy decisions about climate change impacts on *D. opalescens*.

Introduction

D. opalescens is a North-eastern Pacific inshore squid ranging from Baja California Sur, Mexico to Alaska, USA (Okutani and McGowan 1969, Vojkovich 1998, Jareb et al. 2010). Essential habitat is defined by the USA Magnuson-Steven Act as “those waters and substrate necessary to fish (federally defined as including squid) for spawning, breeding, feeding, or growth to maturity.” In the Southern California Bight (SCB), *D. opalescens* lay their embryo capsules on the seafloor of the continental shelf (< 100 m depth; McGowan 1954, Okutani and McGowan 1969, Zeidberg et al. 2011a). *D. opalescens* have been predominately observed to lay embryo capsules onto sandy habitat (Zeidberg et al. 2011a) but do not have any strong affinity towards any particular type of sandy substrate (Young et al. 2011), possibly indicating that sand itself is not a cue. Geologically, the SCB has expansive sandy plains on the shelf (Revelle and Sheppard 1939) making these habitats more available to *D. opalescens* compared to other habitats. Thus, *D. opalescens* may not actively seek sandy substrate.

Given that *D. opalescens* is a vital forage species (Morejohn et al. 1978) of the California Current System and a major commercial fishery (Porzio 2013), the lack of understanding of essential embryo habitat is surprising. Economic exploitation of *D. opalescens* started in Monterey in the 1850s by Chinese immigrants and then expanded to Italian immigrants until it became a major fishery in the 1970s (Vojkovich 1998, Brady 2008). Since the 1990s, the commercial fishery has boomed in the SCB (Vojkovich 1998, Porzio 2013). Over the past four years the market squid fishery (*D. opalescens*) has met the annual quota of 107,030 m tons and the fishery has closed early (Porzio 2013). Not only is *D. opalescens* the main species of squid harvested in California, USA (Vojkovich

1998), this single species constitutes up to 60% of the entire biomass of all the marine species captured (Porzio 2013). The trend of the California commercial fishery take is consistent with previous predictions for an increasingly important invertebrate harvest as part of what investigators called, “Fishing down marine food webs” (Pauly et al. 1998, Pauly and Palomares 2005). According to the Magnuson-Stevens Fishery Conservation and Management Act (Coppes et al. 2007), understanding “essential” habitat is mandated by USA federal law for fishery species and adaptive management procedures have been put in place to incorporate new science as it is acquired (Henry et al. 2005).

The Inner-shelf Environment of the Southern California Bight

D. opalescens embryo capsules in the Southern California Bight (SCB) can be found in high abundance between 11-14 °C (Zeidberg et al. 2011a). It is unknown whether adults are selecting their spawning sites based on temperature or on other environmental factors (e.g. oxygen, pH, $p\text{CO}_2$, nutrient concentrations, light, current velocity, etc.). It is also unknown whether or not *D. opalescens* embryos are inhibited by low temperature, $[\text{O}_2]$, and pH (and high $p\text{CO}_2$) exposure in nature, but it is known that embryo development is affected by temperature (Ziedberg et al. 2011b), $[\text{O}_2]$ and pH (Chapter 3) in the laboratory. In the SCB, where the majority of *D. opalescens* commercial fishing occurs (Porzio 2013), pH and $[\text{O}_2]$ dramatically decrease with water depth in areas utilized by *D. opalescens* embryos. pH and $[\text{O}_2]$ are predicted to decrease at these depths into the foreseeable future due to changing climate (Gruber et al. 2012, Takeshita et al. 2014).

To date, the surface waters in the California Current System have declined by 0.1 pH units since the industrial revolution began (Hauri et al. 2009) and the oxygen minimum zone upper boundary has shoaled over the last 25 years (Bograd et al. 2008, 2014, McClatchie et al. 2010, Booth et al. 2012, 2014). Further, evidence in the SCB suggests that seawater chemistry is increasingly dynamic over decadal (Bograd et al. 2008, McClatchie et al. 2010, Booth et al. 2014), yearly (Nam et al. 2011), seasonal (Send and Nam 2012), weekly and daily (Frieder et al. 2012) scales. Upwelling in the CA current is intensifying (Sydeman et al. 2014) and oxygen (and pH) declines are especially severe inshore (Bograd et al. 2008), particularly within 10 km of shore (Booth et al. 2014). Species that are susceptible to habitat compression with climate change may “lose” as the environment changes. Declines in [O₂] and pH can compress rockfish habitat (McClatchie et al. 2010) and retard development of mussel larvae (Frieder et al. 2014) and squid embryos (Navarro et al. 2012; Chapter 3).

Doryteuthis opalescens

Fishery-independent surveys of *D. opalescens* in the SCB have not been conducted since the 1950s (Okutani and McGowan 1969, McGowan 1954). However, recent surveys of the commercially fished areas show that areas utilized by squid embryos (Zeidberg et al. 2011a) are likely exposed to waters containing low [O₂] and pH. If so, impacts on the squid population could also affect downstream ecosystem interactions. *D. opalescens* is a forage species and is an energetically integral part of the ecosystem both as a predator as well as an abundant prey item for fish, sea birds and marine mammals (Morejohn et. al 1978).

D. opalescens is a fast growing species with a short generation time; their life cycles are shorter than one year (Yang et al. 1986, Jackson and Domeier 2003). Spawning age of squid is highly dependent on oceanographic conditions (e.g. El Niño Southern Oscillation (ENSO)) and ranges from ~4.5 months old (129-137 d) during La Niña to ~8 months old (225-257 d) during El Niño (Jackson and Domeier 2003). Presumably squid spawn as soon as possible to avoid the mortality risk that occurs while “waiting” to mate. After mating, *D. opalescens* die (a semelparous life history strategy; Fields 1965); death has been estimated to occur within two weeks of spawning (Perretti 2014). Quick growth rate and short generation times may allow *D. opalescens* populations to respond rapidly to changes in oceanographic conditions (Jackson and Domeier 2003, Zeidberg et al. 2004, Koslow and Allen 2011). Further, *D. opalescens* may function as “ecosystem recorders” or provide integrated measure of oceanographic conditions (Jackson and Domeier 2003).

In this chapter, *D. opalescens* use of the northern La Jolla coastline, USA (hereafter referred to as the McGowan Site; 32.86 °N, 117.28 °W), will be explored in the context of changing [O₂] and pH in the SCB. This area was chosen because of a long history of scientific observation of *D. opalescens* embryos (McGowan 1954, Okutani and McGowan 1969) and of the environment at SIO (McGowan et al. 2010).

Microcohort Analysis

Post-spawning adult statoliths are investigated to examine microcohort characteristics. A microcohort is a group of squid, within a subpopulation, that spawns at a distinct time period from other groups of squid. Microstructural analysis of statoliths

has been shown to be a robust method to estimate age for individual *D. opalescens* (Yang et. al 1986, Jackson 1994a), allowing analysis of microcohorts. Statoliths are a biogenic carbonate (aragonite) structure found within the statocysts and statocysts are paired and are used by squid to detect vertical and horizontal acceleration (Clarke 1978, Jackson 1994a, Arkhipkin and Bizokov 2000). Statoliths have been shown to contain “dark and “light” rings that together form a daily growth increment (Yang et. al 1986, Jackson 1994a). In addition, statoliths contain a very dark hatch mark, which can be used to determine size at hatching (Ceriola and Jackson 2010). Temperature variation, development time, and food availability can directly affect the microstructure of statoliths. Statolith ring microstructure (e.g., growth) can be analyzed as a calcified record of the developmental history of that individual squid (Jackson 1994b, Ceriola and Jackson 2010). Consequently, statolith microstructure (ring numbers) can be used to compare age composition between two spawning *D. opalescens* groups to determine whether or not they have distinct birth dates.

Studies have shown that there are differences between size, growth rates and age of market squid during years with La Niña conditions compared to El Niño years (Jackson and Domeier 2003, Zeidberg et al. 2004). Most research has been done on response to annual changes in water temperature and food availability (Jackson and Domeier 2003, Zeidberg et al. 2004, Jackson et al. 2010). The large and variable period of maturity of squid may allow for timed spawning according to favorable oceanographic conditions. The objective here is to test for age differences between temporally separated groups of spawning *D. opalescens* (post-spawned squid). This can be tested among

groups temporally separated by weeks because female squid lay most of their embryo capsules within the first three days of spawning (Perretti 2014).

Spawning Site Selection

Little is known regarding how *D. opalescens* selects spawning sites. In the SCB, *D. opalescens* is reported to spawn from October to May (Henry et al. 2005). *D. opalescens* embryo capsules are often observed within narrow temperature (10-14 °C) and depth ranges (20-70 m) and almost exclusively on sandy habitat (Zeidberg et al. 2011a). Further, market squid have long been recognized to repeatedly utilize the same geographic area/habitat each spawning “season” (Fields 1965, Vojkovich 1998, Zeidberg and Hamner 2002). *D. opalescens* may be invoking traditions presumably from endogenous individual responses to arbitrary environmental features. Traditions are defined as social behaviors that extend across generations (Warner 1990). Salmon are an example of an organism that uses an arbitrary environmental feature to spawn (e.g. chemical cue). The importance of tradition vs. individual assessment is unknown but, for many species a mix of approaches is expected (Warner 1990). Spawners benefit from adopting a locally adaptive phenotype. *D. opalescens* repeatedly uses the same site for deposition of embryo capsules. This is evidence that these squid, at least in part, use tradition to select their spawning site. However, utilizing tradition can be costly when environmental changes associated with traditional spawning sites are detrimental to offspring (Warner et al. 1990). As such, most organisms utilize a mix of tradition with individual assessment.

In the scenario where environmental change associated at a traditional-spawning site is costly to offspring, individual assessment is more advantageous than tradition. Although this area of research is clearly data poor for *D. opalescens*, compelling clues as to the cues females use to select sites to lay their embryo capsules have been revealed in the laboratory setting. Fields (1965) found that *D. opalescens* consistently lays embryo capsules at or immediately next to the aerated seawater inflow suggesting that high [O₂] and high pH (low pCO₂) are important cues for site selection. Does *D. opalescens* use tradition and/or individual assessment when making the site selection for their embryos? In nature, the cue(s) for *D. opalescens* spawning-site selection is an open and important question key to understanding essential embryo habitat.

The potential available habitat for *D. opalescens* has been estimated to include the majority of the continental shelf in the SCB (Zeidberg et al. 2011a). However, the realized *D. opalescens* embryo habitat has yet to be identified. For example, implicit within the Zeidberg et al. (2011a) description of squid-embryo habitat is the influence of seasonal, event, and daily physico-chemical variation. In the SCB, the seasonal (Hickey 1979, Lynn and Simpson 1990, Checkley and Barth 2009, Send and Nam 2012), event (Nam et al. 2011, Frieder et al. 2012), and daily scales (Frieder et al. 2012) are generally known. However, the actual physico-chemical changes that occur at an embryo bed while embryos are developing are undocumented. This chapter characterizes the seasonal and higher frequency hydrographic variation occurring at the McGowan Site.

Objective and Hypotheses

The overall objective of this paper is to evaluate the essential embryo habitat of *Doryteuthis opalescens* in the SCB. To do this I will: (1) describe where *D. opalescens* occurs with respect to substrate type, depth, and the local submarine canyons, (2) describe *D. opalescens* occurrence with respect to seasonal and annual cycles, (3) synthesize these data to describe microcohort structure in terms of spawning adult age, event duration and embryos•capsule⁻¹, (4) describe the seawater conditions ([O₂], pH, T and S) of the embryo beds and consider associated consequences and (5) make recommendations for commercial fisheries in southern California, USA and for MPA managers. This work tests the null hypotheses that: (1) *D. opalescens* does not exhibit tradition (defn. Warner 1990) and (2) *D. opalescens* microcohorts spawn continuously. (3) Environmental conditions are similar both when embryos are present compared to when they are absent (in space and time) and (4) abundance, age at spawning, and number of embryos •capsule⁻¹ are similar among microcohorts.

Methods

Study Area

Prior to large changes in the CA commercial fishery, the *D. opalescens* embryo habitat at the McGowan site (Figure 2.1.) was directly observed in March, 1953 (McGowan 1954). In December 1954, the largest recorded squid-embryo bed ($1.76 \cdot 10^{12}$ embryos) was estimated to cover an area spanning $1.6 \cdot 10^7 \text{ m}^2$ (Okutani and McGowan 1969). The SIO pier station has provided long-term data for this area since 1916 including temperature, salinity, and density (McGowan et al. 2010). This site is also

located next to the Del Mar mooring (9 km NNW, 32.93 °N, 117.31°W), which at present continuously monitors pH, [O₂], as well as chlorophyll, salinity, temperature, and density for the water column surface, at 35 m and at 88 m (described in Nam et al. 2011). The McGowan site is of historical importance and is also one of the most thoroughly observed areas within *D. opalescens* range in terms of physics, chemistry, geology and biology, making it ideal for defining essential embryo habitat.

Environmental Data Acquisition (Aug. 2009 – Oct. 2013)

At the McGowan site (Figure 2.1.), environmental measurements were made using SeaBird casts (salinity, temperature, depth and oxygen when equipped; Figure 2.2.), HOBO-tidbit ® loggers (temperature; Figure 2.3.) and a SeapHOx instrument (salinity, temperature, depth, oxygen, pH; Figure 2.4.; SeapHOx described in Frieder et al. 2012) to determine the environmental conditions of available habitat versus realized squid embryo habitat (Table 2.1.; Figure 2.1.). A CTD instrument cast at La Jolla submarine canyon was made to characterize the canyon water column (Figure 2.2.). In addition, HOBO-tidbit ® loggers were deployed at 25-30 m depth intermittently throughout the observation period (Table 2.1.; Figure 2.3.). Further, SeaBird CTD instruments were attached to ROV Honu (Seabotix 150; Table 2.2.) and ROV Triton (Scripps) to assess environmental conditions near the sea floor. CTD instruments on the ROVs lacked pH sensors. Alin et al. (2012) algorithms were used to estimate pH using [O₂] and temperature measured by ROV CTDs. The SeapHOx was deployed 0.5 m above the seafloor within an embryo bed on the sandy plain within the McGowan Site (Figure 2.1.; Tables 2.1., 2.3.).

Biological Data Acquisition

Three investigations generated biological data: (a) of the upper shelf (< 30 m), (b) the mid and lower shelf (30-100 m depth) and (c) the upper slope (100-400 m depth). For the upper shelf, small boat surveys, using a drop-down camera, and SCUBA surveys were used of to assess spawning and embryo habitat (Table 2.4. and 2.5.; Figures 2.7.-2.9.). Collections of post-spawning adults and embryo capsules were made using SCUBA (see “Demography of Microcohorts” for details).

For the mid and lower shelf investigation, taxon-specific depth and distribution data were collected from ROV/small-boat surveys of seafloor megafauna using ROV Honu with a mounted Seabird CTD and oxygen sensor (Table 2.6.; Figures 2.10.-2.19.). Surveys were temporally stratified to include upwelling (spring/summer) and non-upwelling (fall/winter) seasons. Area of the embryo beds was extrapolated from transects and density was estimated from counts of mops and isolated embryo capsules ($5 \cdot \text{sec}^{-1}$). Mops were graded by size (small, medium, large) with small being estimated as < 5 capsules, medium as 6 - 25 capsules and large as 26 - 125 capsules. Density was averaged over the survey transect and multiplied by the area to estimate total capsule count for the bed. Total embryo abundance was estimated by multiplying the estimated total capsule count by the estimated number of embryos $\cdot \text{capsule}^{-1}$ (see “Demography of Microcohorts” for details about embryos $\cdot \text{capsule}^{-1}$).

The upper-slope ROV surveys were conducted using the Scripps ROV Triton (Table 2.7.; Figure 2.20.). ROV transects were conducted at one location at 400 m depth

during July, 2012, and at two locations each at 100 m, 200 m, 300 m and 400 m depths during December, 2012, as part of the San Diego Coastal Expedition (SDCE). Upper-slope otter trawl surveys were conducted at 100 m, 300 m and 400 m depths during the SDCE (Table 2.7.; Figure 2.20.).

Demography of Microcohorts

D. opalescens were collected on 28 October, 2011 and 11 November, 2011 by hand using SCUBA at the McGowan site. Twenty-five individuals were collected at 26.7 m in October and twelve individuals were collected on November 11. *D. opalescens* were identified as male or female and measurements were made on the dorsal mantle lengths (DML), total weight (TW), and weight of gonads (GW). The nidamental gland, ovaries and oviduct were collected for female gonads and the testis and spermatophoric complex for male gonads. Weights were measured to the nearest hundredth of a gram using an analytical balance. Lengths were measured to the nearest millimeter. To determine if *D. opalescens* were likely to have spawned in a single event or as part of multiple-spawning events, squid gonadosomatic index (GSI) was calculated (with methods adopted from Ceriola and Jackson 2010):

$$\text{GSI} = (\text{total reproductive weight}/\text{total body weight}) \cdot 100$$

Statoliths were removed, rinse in milli-Q water, dried, placed into individual envelopes and stored in a -20°C freezer. Daily ageing criteria methods were minimally modified from Yang et al. (1986) and Jackson (1994a). Left and right statoliths were dissected from squid were dried and stored separately. Statoliths were positioned anterior side up

and mounted onto slides using the thermoplastic cement Crystal Bond. Statoliths were polished first using 30- μm paper and then 3- μm polishing paper until the increment plane (where “rings” were detectable) was reached. Increment counts were made using a compound microscope (Figure 2.21.) with the aid of a hand counter. Two people independently aged each statolith, conducting double-blind counts. Only when the ages by each counter varied by 10% or less was the data used. For these data, the age was determined as the average of both counts. Date of birth was back-calculated using daily growth increments and was determined by subtracting the daily increments from the date of collection (Figure 2.22.).

Post-spawn *D. opalescens* were collected either as dying or dead spawning squid. When dead squid were collected the statoliths became opaque. In these cases, rings could not be detected using microscopy techniques and then indirect aging methods using statolith size measurements were employed (Jackson 1994a). The regression model was produced using statoliths from squid that were reasonably similar, both temporally and spatially, to individual squid with opaque statoliths. The model estimated age from the shortest length of the dorsal dome (natal core to outer edge of the dorsal dome at its junction with the outer edge of the lateral dome).

Collection of squid-embryo capsules were made at the McGowan site via SCUBA on 8 Dec. 2012, 29 Feb. 2012, 3 May 2012, 30 Apr.2013, 2 May 2013, 10 May 2013, 11 Jun. 2013 and on 25 July 2013. Capsules were dissected and embryos were double-blind counted under a dissecting microscope. Results per capsule were used only when counts agreed within 5% of one another.

Results

Substrate and Habitat Selection

In late February, 2009, spawning *D. opalescens* and their embryo capsules were observed at the mouth of the La Jolla submarine canyon from 20-30 m depth (Hawkins et al. 2010; Figure 2.6.). Upon arrival of the commercial fishery in March-May, 2009, *D. opalescens* embryo capsules were observed in deeper waters (25-99 m) south of La Jolla submarine canyon side of the McGowan site. From July 2009 to mid-June 2010 embryo beds were rarely observed above 30 m depth (embryos were seen on 5 Feb, 2010; Figure 2.7.). The lack of observations of *D. opalescens* embryo capsules at these depths coincided with a weak El Niño that began in the summer of 2009 (Bjorkstedt et al. 2010, Nam et al. 2011). Then, concurrently with the arrival of the 2010 La Niña, a large embryo bed was observed at depths of 20-30 m (Figure 2.8.) and embryos were commonly seen at the McGowan site from 23 June, 2010 - 30 July, 2013 (Tables 2.4.-2.6.; Figures 2.8.- 2.19.). At the same time, spawning adults and embryos were not seen on the upper slope in ROV or trawl surveys either in July 2012, or December 2013 (Table 2.7.; Figure 2.20.). Environmental conditions differed during these periods. Reduced [O₂], pH and temperature occur over the shelf according to isopycnal uplift, which can be caused by upwelling events during spring (when upwelling events occur regularly) and during La Niña (Nam et al. 2011). From June 2010 to July 2013, the ENSO state was either La Niña (Summer 2010; Bjorkstedt et al. 2011) or neutral (Spring 2011-Spring 2013; Wells et al. 2013).

To understand the environment of the essential embryo habitat spatially, the ROVs Triton and Honu were used. Squid embryos were only seen on the shelf (< 92 m;

Table 2.4., 5; Figures 2.10.-2.20.). Using ROV Honu (shelf surveys), embryo beds were observed to occur at variable depth ranges with their lower depths having a larger range over time (33.4-91.5 m) compared to the upper depth range (10.3-33.4 m; Table 2.4.; Figures 2.11.-2.19.). *D. opalescens* embryos were found to occur at temperatures, [O₂], and pH that were higher and salinity that was lower than when embryos were absent ($P < 0.0001$ for each environmental factor, Wilcoxon test; Table 2.2.). The shallow portion (< 50 m depth) of the habitat was observed to have *D. opalescens* embryo beds during each survey (9 of 9) whereas the deeper portions (≥ 50 m) had embryo beds only a little more than half of the time (5 of 9; Table 2.4.). *D. opalescens* were concentrated within the oxygen and pH maximum (Table 2.4.) and carbon minimum zone (here after referred to as the oxygen maximum zone) during each ROV survey. The densest areas of the *D. opalescens* embryo beds were usually in (6 of 9) or a few m below in water depth of this zone (2 of 9; Table 2.6.). The O₂/pH maximum was highly variable among the surveys ([O₂]: 160.2-276.9 μ M, pH:7.81-8.11; Table 2.6.). These findings are consistent with anecdotal evidence from the laboratory setting that *D. opalescens* selects aerated portions of aquaria for embryo deposition (Fields 1965) and suggest that *D. opalescens* is using site selection based on high [O₂] (and/or high pH/low $p\text{CO}_2$). One important exception occurred on 26 June 2013, when the densest embryo beds were 8.4 m deeper than the oxygen maximum zone.

To understand the environment of the essential embryo habitat on the temporal scale, temperature loggers and a sea pH_{Ox} were deployed near the seabed at 30-m depth. The depth was chosen because it was within the previously reported depth range of embryo beds in the SCB (Zeidberg et al. 2011) and to allow for SCUBA divers to retrieve

and deploy equipment rapidly. ROV surveys reveal that this depth usually (if not always) has higher pH, [O₂] and temperature and lower salinity levels compared to deeper depths, including the mid and lower shelf and upper slope. At 30 m depth, temperature, [O₂], and pH were lower and salinity was higher when *D. opalescens* was observed ($P < 0.0001$ for each environmental factor, Wilcoxon test; Table 2.3., Figure 2.5.). This suggests an opposite pattern than found in the spatial surveys.

The apparent temporal and spatial patterns mismatch may be due several reasons but may be simple due to squid utilizing the habitat differently over time. Only one depth was studied over time. The 30 m depth is significant among other depths within the squid embryo habitat because temperature, [O₂] and pH are usually higher (if not always higher) compared to deeper depths. From the ROV surveys, *D. opalescens* appears to use the entire shelf at times when the isopycnals are relaxed (as was observed in August 2012). This corresponds to the highest values of temperature, [O₂], and pH at the 30 m depth. Squid embryo capsules were observed over the broadest depth range and as “singles,” with mops or groups of capsules rarely observed. Squid may not be observed at 30 m depth when this occurs is because squid may use the upper shelf (≤ 30 m depth) for spawning when deeper habitat is suitable. That is, one hypothesis is that squid utilize the upper shelf only when [O₂], and pH are too low at the mid and lower shelf. This pattern would also be consistent with the ROV surveys. If so, squid may prefer to avoid the upper shelf for spawning to avoid many of their predators observed in shallow waters including marine mammals, birds and many of the obligate residents of the kelp forests (Morejohn et al. 1978). In contrast, when isopycnals are uplifted, the mid and lower shelf (30-100 m depth) are exposed to low temperature, [O₂], and pH (and high salinity; Nam

et al. 2011) and may be avoided by spawning squid. If so, the squid essential embryo habitat would be compressed from below, causing squid to utilize the shallow portion of the available embryo habitat, including the 30 m depth. The embryo capsules would be relatively dense because of the habitat compression which would also make detection of embryo capsules is easier. Future studies could test these hypotheses by deploying instruments at multiple depths inclusive of the mid and lower shelf (30-100 m depth) and the upper slope (100-300 m depth).

Substrate Selection

D. opalescens attached their embryo to various soft and hard substrates during the study. Soft substrates were limited to coarse sand where squid used an adhesive substance at the base of their embryo capsule to agglutinate sand beneath the sediment surface (1-2 cm depth). However, even when *D. opalescens* attached their embryos to the sand, it was common for other *D. opalescens* to attach capsules to other capsules as has been observed in previous studies (McGowan 1954, Fields 1965). Most hard substrate observations of embryo capsules were on submarine canyon walls when canyons could be observed (all 5 ROV surveys over the canyon: Figures 2.15.-2.19.). *D. opalescens* attached capsules to hard substrate directly using the adhesive properties at the base of the capsule. *D. opalescens* attached capsules on horizontal and vertical surfaces on rock, but often capsules were observed within rock crevices. *D. opalescens* can attach their capsules to kelp stipes, to ropes and to fiberglass materials that were used to mount the SeapHOx to the seafloor (Navarro personal observation). *D. opalescens* embryo capsules were attached to sediments on the sandy substrate during all McGowan

site surveys as well as the survey of Point Loma and Solana Beach (Table 2.4.). Densely packed embryo capsules were usually observed on sandy plains and submarine canyon walls but not on other substrates (Table 2.4.).

In this study, *D. opalescens* did not attach capsules to kelp near the northern La Jolla kelp forest (Figure 2.6.-2.19.) although observations were made of embryo capsules attached to kelp strands outside of the forest (Navarro personal observation). Further, there was an ~50 m-wide zone over the sand adjacent to the kelp forest that was also largely unoccupied by the *D. opalescens* even though this habitat is similar to other embryo habitat (Figure 2.8., 2.14.-2.19.); presumably the squid avoid the area for fear of being eaten or *are* eaten by their predators associated with the kelp forest.

Squid embryo capsules also were not commonly observed within *Dendraster excentricus* (sand dollar) beds, although the squid and sand dollars both occupy similar habitats (Figures 2.9., 2.16.-2.19.). Within *D. opalescens* embryo habitats, *D. excentricus* was rare, and vice versa. Only 0.4% of all *D. opalescens* capsules were observed in sand dollar beds. Further, in sand dollar beds, *D. opalescens* capsules were only seen as single-isolated capsules and their co-occurrence constituted 6% of all the sites where *D. excentricus* were observed. *D. opalescens* either actively avoids laying embryo capsules within *D. excentricus* beds or *D. excentricus* disturb and directly dislodge squid capsules after they are deposited. *D. excentricus* constantly move within and on top of the sandy surface changing the structure of the sand (Morin et al. 1985). Regardless of the mechanism, these two species appear to have a strong competitive interaction.

When squid spawn

D. opalescens embryos occurred at the McGowan site during all five years (2009-13) and through all seasons (Winter 2009-2013; Spring 2009-2010, 2012-2013; Summer 2010-2013 13; Fall 2010-2012; Table 2.4.) during this study period (Spring 2009 - Summer 2013; Tables 2.4.-2.7.). On March 15, 2010 a drop-down camera was utilized and towed using a small power boat, increasing the observation area but was limited to depths ≤ 30 m (Figure 2.7.). On June 25, 2010 *D. opalescens* embryo beds were observed over a large section of the sandy plains, just south of the La Jolla submarine canyon (Figure 2.8.). *D. opalescens* embryos were not observed in Summer 2009, Fall 2009 or Spring 2011 however, observations >30 m depth were only possible with the use of ROV Honu and Triton after June, 2012 (Tables 2.6., 2.7.). It is possible that *D. opalescens* embryos were present during these times even though they were not detected in this study. Overall, this study provides evidence that *D. opalescens* spawns throughout the year.

Microcohort Demographic Structure

A total of 37 individuals were sampled from the McGowan site between 28 October and 11 November, 2010 in order to test whether spawning squid separated by two weeks originated from the same cohort (i.e., were hatched at the same time), or exhibited demographic differences. In the October group, 24/25 individuals were female and in the November group only 5/12 were female. The average dorsal mantle length (DML) for the October group trended longer, 3.3%, than the November group ($t(35) = 1.53$, $P = 0.14$). However, the total weight for the October group was distinct 35.8 %

bigger (10.8 g; $t(35) = 3.43$, $P = 0.0033$) with their gonads weighing 9.6 g more compared to the November group ($t(35) = 12.2$, $P < 0.0001$). The GSI was 2.5 fold higher in the October group and was distinct (GSI=27.3; $F_{1,2}=16.2247$, $P < 0.0001$; two-way ANOVA) compared the other group (GSI = 11.7). The GSI numbers are within the range reported for the species (Fields 1965) and the November females presumably had laid more embryos before they were collected. Males had similar GSI (although only one male was sampled for October).

The back-calculated average birth date of adult squid collected in October was June 16, 2010; for those collected in November it was June 12, 2010 (Figure 2.23.). The squid from October ranged in age from 122-146 days with an average of 135 ± 2 days. The November group had an age range of 145-170 days with an average of 152 ± 2 days.

Collections of embryo capsules were made throughout the observation period (Figure 2.23.). Embryos•capsule⁻¹ averages were distinct among collection groups ($F_{1,7} = 3.77$, $P = 0.0017$) indicating seasonal differences. Data from these collection groups roughly fit a quadratic regression ($R = 0.258$). This regression was used to estimate embryos•capsule⁻¹ for each date surveyed using the ROV. Then ROV survey estimates of total capsules were multiplied by the embryos•capsule⁻¹ estimate to calculate the total embryo abundance for each embryo bed surveyed. Total abundance of the embryos of each bed surveyed varied by three orders of magnitude (Table 2.4.). The highest estimated total embryo abundance within any embryo bed surveyed in this study was $5.2 \cdot 10^9$ embryos. Still, this estimate is three orders of magnitude less than that made for December, 1954 (Okutani and McGowan 1969). Part of this discrepancy might be due to

the December, 1954 embryo bed covering a 3-fold larger area. Additionally, our estimate of number of capsules $\cdot \text{mop}^{-1}$ in this study was conservative and is probably an underestimation.

Discussion

Spawning

Observations from February 2009 – July 2013 provide direct evidence consistent with *D. opalescens* spawning in “waves” as has been inferred previously through statolith analyses (Jackson and Domeier 2003) and from the GSI of female squid (Fields 1950). Further, this study provides direct evidence that *D. opalescens* spawning can occur throughout the year over a relatively small geographic area (relative to the adult range). Statolith analyses of the sister taxon, *D. pealeii*, support a similar spawning behavior (Brodziak and Macy 1996, Macy and Brodziak 2001) and indicate that the waves of spawning are consistent in structure with a “continuum of microcohorts” that has been found for another loliginid, *Sepioteuthis australis* (Moltschaniwskyj and Pecl 2007). Collectively, these studies suggest that this type of spawning behavior may be pervasive throughout the 40 species within the Loliginidae family (Anderson 2000) although it has not been examined for the vast majority.

This study shows that *D. opalescens* size and age at reproduction can vary within a season at La Jolla, USA. The age structure of two groups of spawning *D. opalescens* at the McGowan site offers a quantitative means to study the spawning and response behavior of this species on a monthly time scale. *D. opalescens* are back-calculated to

have hatched over the same month long period, although they spawned and died at different times. Females dominated the October group, which is consistent with their dying first and with findings in the laboratory setting (Perretti 2014). This was also reflected in the GSI index which was higher for the October group.

The back-calculated birth dates were compared to the seafloor temperature at the McGowan site (Figure 2.22.). It is interesting that the younger squid spawned first and this may be evidence that the environment plays a role in squid development on smaller spatial and temporal scale than previously documented (Ex. Environmental difference within the SCB over a period of 6 months). Since only two squid hatched after 23 June, prior to the start of the La Niña (Figure 2.22.), it is possible that these squid either developed in waters at the McGowan site but deeper than 30 m depth or that these squid developed at another site. Earlier studies have determined that *D. opalescens* responds to oceanographic conditions on an annual and seasonal time-scale (Butler et al. 1999, Jackson and Domeier 2003, Zeidberg et al 2004, Jackson et al 2010). The implication of this result is that females spawn first and that spawning takes at least two weeks. Since this is the first evidence of age and size structure of *post-spawned* squid within a season, more testing should be conducted at both small and large temporal and geographic spatial scales for *D. opalescens*.

Age Structure

The range of ages reported in this study is within but near the low end of the range of ages reported for mature *D. opalescens* (Jackson and Domeier 2003, Butler et al. 1999). This may be indicative of a rapid response to a La Niña. Starting in the summer of 2010, strong La Niña conditions were present in the California Current Ecosystem (Bjorkstedt et al. 2011) and lasted until the spring of 2012 (Bjorkstedt et al. 2012). Previous studies have found that market squid respond to food availability and temperature variation through accelerated growth rates and earlier maturation rates (Butler et al. 1999, Zeidberg et al 2004, Jackson et al 2010) on an annual, semi-annual and seasonal time scales (Butler et al. 1999, Zeidberg et al 2004). Current management of the market squid fishery restricts commercial harvest to an annual quota (107,030 metric tons). This study found that spawning market squid differed in age and biomass over period of weeks.

Fishery Management: Consideration of a dynamic shelf environment in defining essential embryo habitat

The market squid fishery management plan (MSFMP, Henry et al. 2005) states that, “The Commission may take four general types of actions within the framework of the MSFMP: 1) FMP amendment, 2) full rulemaking, 3) notice action, and 4) prescribed action.”

This study suggests that *D. opalescens* commonly spawns continuously on the shelf in the SCB, that spawning behavior is indicative of a tradition (defn. Warner 1990) as there is repeated use of the McGowan site, squid-embryo distribution is strongly associated with exposure to high [O₂] and pH (low pCO₂) and spawning squid may be cueing to better aerated waters. Evidence suggests there are strong predatory and competitive interactions along the upper boundary of embryo habitat, and preliminary data show that females spawn first. Prior to this work, the MSFMP acknowledged that aging data “*strongly suggests that a new cohort*” spawns “*almost monthly*” (Section 1-20), but spawning was thought to *usually* occur at different geographic locations throughout the year (Section 1-21; Henry et al. 2005). The plan indicates that southern California spawning occurs from October – April or May, whereas central California spawning occurs from April to October, and attributes this to temperature difference. It also recognizes that in some years spawning may occur throughout the year, reducing effects of poor local conditions and limiting importance of stock from a single area (Henry et al. 2005).

The MSFMP acknowledges large knowledge gaps for *D. opalescens*. *D. opalescens* spends a considerable amount of time at or near the seafloor during its life cycle; however, beyond this study information is largely limited to one-time sampling. This study provides evidence that *D. opalescens* does not utilize all areas of the shelf and slope similarly and that the environment appears to be important in both how *D. opalescens* selects its spawning sites as well as how well embryos are able to develop. The MSFMP policy mandates an “adaptive” strategy to allow flexibility as the scientific knowledge for this species increases. This is especially important considering the

growing knowledge surrounding climate change impacts on cephalopod species (Robin et al. 2014). There is growing evidence in the SCB that suggests that seawater chemistry (T, O₂, pH, pCO₂) is increasingly dynamic over decadal (Bograd et al. 2008, McClatchie et al. 2010, Booth et al. 2014), yearly (Nam et al. 2011), seasonal (Send and Nam 2012), weekly and daily (Frieder et al. 2012) time scales. How variations on each time scale affect squid dynamics remains to be determined.

This study provides several key findings for management. A notice action is recommended to map essential embryo habitat and frequency of spawning throughout the range of *D. opalescens* and to evaluate seawater chemistry (T, O₂, pH, pCO₂) at several spawning sites within of each of the three regions of the CCS (Checkley and Barth 2009): This is based on the findings that the McGowan site is utilized by a continuum of microcohorts spawning throughout the year at a *small geographic location*. The core “realized” habitat is surprisingly small at the McGowan site ($\sim 3.7 \cdot 10^6$ m²; Figure 2.15.) and this may be a prominent characteristic of *D. opalescens* spawning sites in SCB. The repeated migration to this site by *D. opalescens* is an expression of tradition which organisms utilize to avoid “trial and error” costs; however, tradition is costly when rapid environment changes associated with traditional spawning sites are detrimental to offspring (Warner et al. 1990). This study shows that the chemical environment can rapidly change to levels that can be detrimental to offspring (Figure 2.18.; Chapter 3).

At the McGowan site, *D. opalescens* site selection for embryo deposition is consistent in cuing to “oxygen maximum zones.” Further, a positive feedback loop is maintained by squid laying capsules on other capsules (Fields 1950, Fields 1965, Okutani and McGowan 1969) to utilize this “oxygen maximum zone.” To a certain extent,

spawning adults can mitigate negative impacts of exposure to low oxygen and pH by avoiding deeper areas of the shelf (Table 2.4.). Although this strategy is beneficial most of the time, the environment changed quickly over the summer in 2013 (after squid laid their embryo capsules). *D. opalescens* embryos may be negatively impacted by low-oxygen levels when immersed beneath a sharp oxycline, as occurred on 26 June, 2013 (Figure 2.18.).

Further, the upper boundary of squid embryo habitat is highly impacted by biological interactions that are currently understudied, with this study providing the only description known. Specifically, *D. opalescens* predators may prevent (directly or indirectly) *D. opalescens* from utilizing area within *or near* kelp forests. In the SCB, this will likely be an increasing challenge for *D. opalescens* as seawater is predicted to become more acidic (Takeshita et al. 2014) and if past trends continue, less oxygenated (Bograd et al. 2014, Booth et al. 2014). *D. opalescens* only can move into shallower water and is likely already experiencing habitat compression as is occurring for some types of rockfish (McClatchie et al. 2010) and myctophids (Koslow et al. 2011).

Climate Cycles

As research continues to fill large knowledge gaps regarding *D. opalescens*, the market squid fishery continues to boom (Porzio 2013). La Niña is often associated with isopycnal uplift where most of the shelf is exposed to low [O₂] and pH (Nam et al. 2011, Send and Nam 2012). The concentration of *D. opalescens* embryos within oxygen maximum zones found here would suggest that during La Niña, adults are also concentrated at these zones because these zones shoal during La Niña. Although squid

are thought to be most abundant during La Niña (Reiss et al. 2004, Koslow et al. 2011, Dorval et al. 2013) determining population size using spawning aggregations data (or those data highly associated with spawning aggregations, such as paralarvae data) can be difficult to accurately quantify (Erisman et al. 2011). Further, habitat compression has not been considered for these population estimates further adding to the “illusion of plenty.” Often the fishery will “bust” in response to El Niño and then immediately “boom” in response to La Niña (Reiss et al. 2004, Zeidberg et al 2004). Work conducted here supports the prediction that, during an El Niño, a broad region on the shelf would be well oxygenated over much deeper depths, compared to La Niña. *D. opalescens* might be cued to deposit embryos over a much broader area. Certainly, the decline in fisheries during El Niño is thought to be associated with a decline in squid abundance (Koslow et al. 2011, Dorval et al. 2013). Work conducted here suggests that these estimates might underestimate squid population because squid may be spawning over a wide depth range and over a broad area making them harder to detect and observe. One example provided in this chapter is the survey at the McGowan site on 30 August, 2012 (Figure 2.11.).

Future studies should focus on deoxygenation and acidification impacts on *D. opalescens*. Along with parallel laboratory studies: (1) Studies should address how susceptible *D. opalescens* spawning adults are to making site selection “mistakes” for their embryos. How often are locations chosen for embryo deposition exposed to poor oceanographic conditions? In this study, the oxygen maximum zone was deep (19.1-26.1 m) on 14 June 2013 but then shoaled on 26 June 2013 (13.3-19.4 m; Table 2.4., Figures 2.4., 2.17.). How often does this happen and is the rate of occurrence increasing? Which sites are most resilient to low O₂ and pH (high pCO₂) events? Studies should evaluate

how resistant *D. opalescens* embryos are to low oxygen. Monthly (chronic) exposure causes negative effects (Chapter 3) but what about over upwelling time scales (3-10 d)? Further, which embryo stages are most susceptible? Does variation on daily time scales alleviate effects of low average oxygen levels?

Ecosystem Linkages

D. opalescens embryo abundance (Table 2.4.) is directly related to spawning adults. Spawning adult abundance tracks the biomass of zooplankton (*D. opalescens* prey). In addition to being an integrated oceanographic measure (Jackson and Domeier 2003), *D. opalescens* frequency and embryo abundance might be an integrated measure of zooplankton abundance. When food is plentiful, the squid then spawn at a younger age (Jackson and Domeier 2003) resulting in more frequent and larger embryo beds. This biomass of spawning squid can result in a “quick” energy transfer to higher trophic levels in the ecosystem. The down-stream effects of high *D. opalescens* biomass could benefit taxa from higher-trophic levels. This might be especially important for predators that heavily rely on squid for prey such as California sea lions, *Zalophus californianus*. When squid are accessible (dense and < 98.2 m from the surface) nursing sea lion moms can do a better job feeding their pups (Lowry and Carreta 1999). Impacts on *D. opalescens* may affect other guilds, such as the large fishes, but these impacts are much more uncertain. The site selected by *D. opalescens* spawning adults for embryo deposition determines the location of key energy transfers on the shelf. It is unknown how important these migrations are to the inshore ecosystems because until now the frequency of these migrations was unknown.

D. opalescens utilizes the shelf as a vital area for incubating embryos and although these areas may already be nutrient rich, the large biomasses of dead post-spawned adults may be an important “seeding” mechanism. That is as these microcohorts are eaten and/or decompose they may add an important source of nutrients to the ecosystem to promote phytoplankton blooms and eventually zooplankton population growth. Although a developmental hypothesis has yet to be tested, it is interesting that these squid spend so much of their life in the embryonic stage. Depending on environmental conditions, 10-25% of the entire life span of *D. opalescens* is spent in the embryonic life stage. The evolutionary reasons a long embryogenesis duration are still not well understood but may hint at a timing mechanism for hatchlings to successfully hunt prey (copepods).

The oceanographic conditions that benefit the *D. opalescens* juvenile and adults may not benefit their offspring (this is a common characteristic for migrating spawners such as salmon). *D. opalescens* adults commonly have abundant prey when nutrients are high but *D. opalescens* embryo habitat is reduced (vertically compressed) as [O₂] and pH are low. To a certain extent, spawning adults can mitigate negative impacts of exposure to low [O₂] and pH by avoiding deeper areas of the shelf. Still, spawning adults can make “mistakes” in their site selection and when they do the embryos develop in very harsh conditions. On the other hand, *D. opalescens* spawning adults can make mistakes by migrating to areas that are too shallow or to areas where there are unfavorable biotic interactions. These include areas high in *D. opalescens* predators such as near or within kelp forest habitat or within areas where embryo capsules are likely disturbed, such as

sand dollar beds. Even less studied are possible mutualistic interactions of embryo capsules with bacteria and diatoms. The realized squid embryo habitat mapped in this study likely represents those areas that have favorable biotic interactions as well as an optimal environment.

Summary and Conclusions

Spawning aggregations of *D. opalescens* at the McGowan site (SCB) occur as a “continuum of spawning microcohorts” as has been reported for *D. opalescens* at Santa Catalina Channel Island, USA (Jackson and Domeier 2003) and in Monterey, USA (Fields 1950). Similar spawning behavior has also been found for the sister taxon *D. pealei* (Brodziak and Macy 1996, Macy and Brodziak 2001) as well as another loliginid, *Sepioteuthis australis* (Jackson and Pecl 2003, Moltshaniwskyj and Pecl 2007).

Presumably continuous use of the shelf for *D. opalescens* spawning is common throughout the SCB (Jackson and Domeier 2003) and perhaps even throughout the range of *D. opalescens* (Fields 1950). *D. opalescens* belongs to family of 40 species, Loliginidae (Anderson 2000), and most of the spawning patterns for these species lack rigorous study. However, two other species have been described to spawn like *D. opalescens* including *Sepioteuthis australis* (Jackson and Pecl 2003) and *D. pealei* (Brodziak and Macy 1996, Macy and Brodziak 2001). Thus, this type of strategy could be a common characteristic to Loliginidae (Jackson and Domeier 2003).

Work conducted here defines *D. opalescens* essential embryo habitat as areas traditionally selected by *D. opalescens* spawning adults for embryo deposition that have

relatively high oxygen and pH levels (minimum $p\text{CO}_2$ levels; Figure 2.24.). This may be the mechanism explaining why squid migrate to spawn on the shelf, even though this area has dense predators. Further, work from this chapter supports that squid are most easily detected (and harvested) when their habitat is compressed (upper shelf, < 40 m depth). Precautionary principles should be invoked to protect essential embryo habitat, especially during times when the seawater has low $[\text{O}_2]$ and pH (e.g. upwelling events, spring and summer, La Niña). Squid spawning aggregations, like all spawning aggregations, may be susceptible to the “illusion of plenty” (Erisman et al. 2011).

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Figures

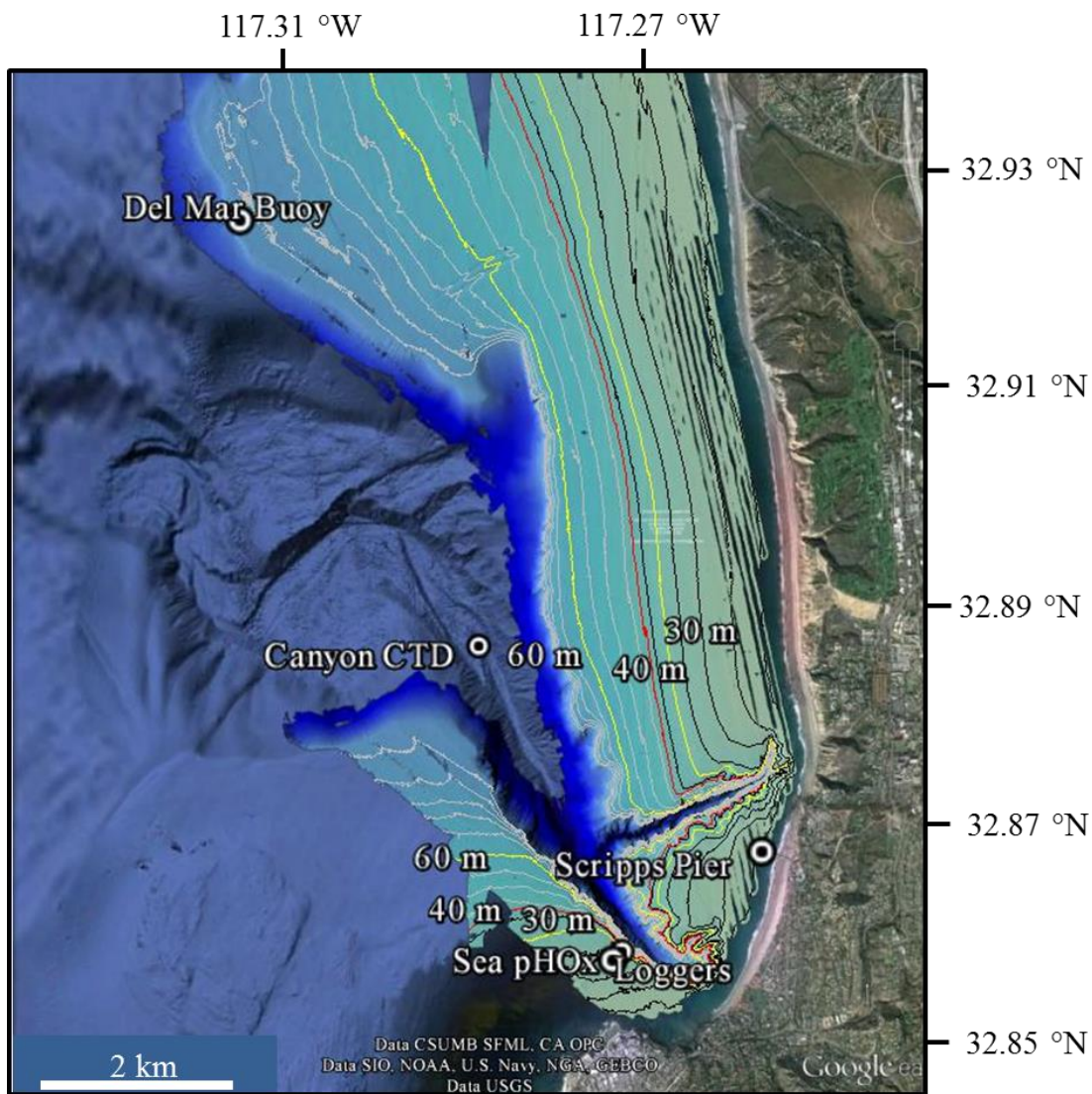


Figure 2.1. Stations for all instruments used during the observation period (18 Aug, 2009 – 4 July, 2013).

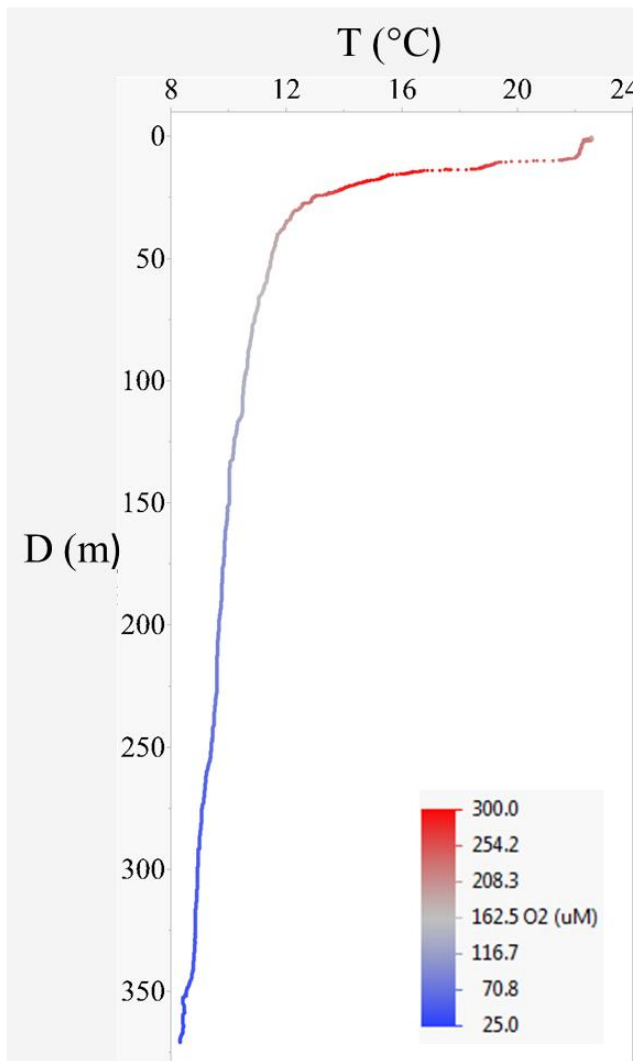


Figure 2.2. CTD cast taken on 18 August, 2009 at La Jolla Submarine Canyon, USA. A. Depth profile of temperature color coded with associated $[O_2]$. Oxygen maximum zone ($> 250 \mu\text{M}$) was found from 19.4-23.7 m depth.

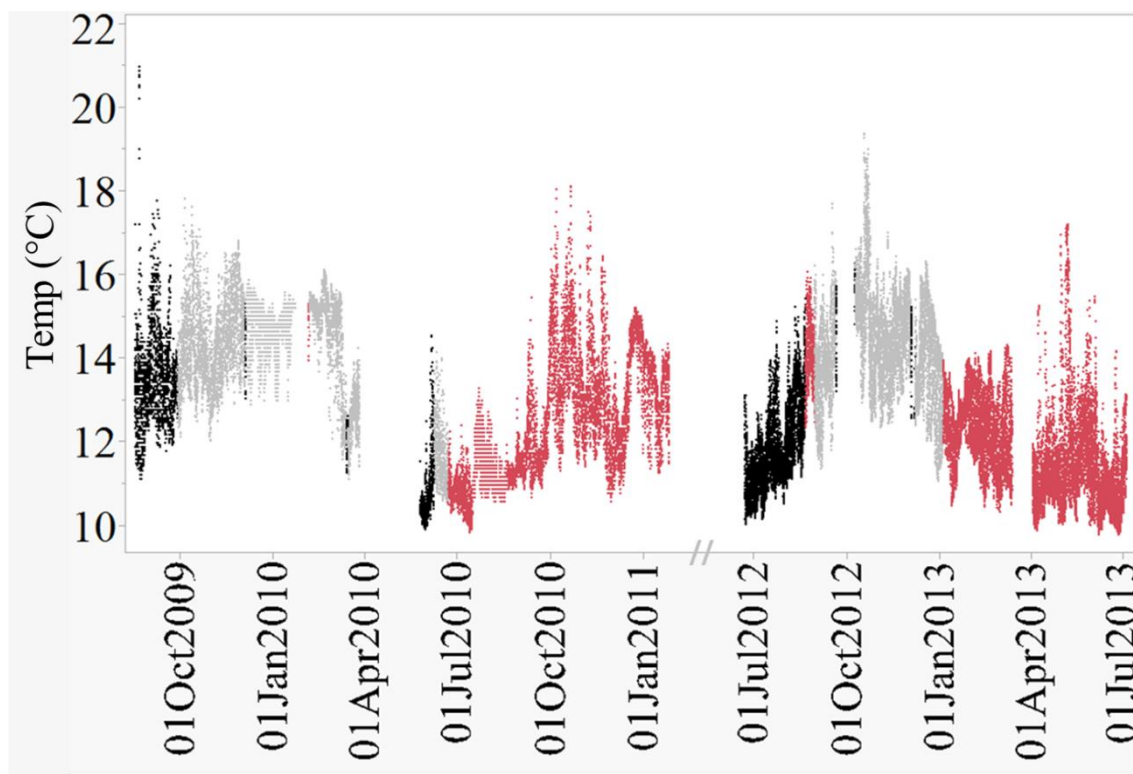


Figure 2.3. Seafloor temperature at the McGowan site (32.86 °N, 117.28°W). Depth=25-30 m. Black = *D. opalescens* absent, Grey = *D. opalescens* not surveyed, Red = *D. opalescens* present.

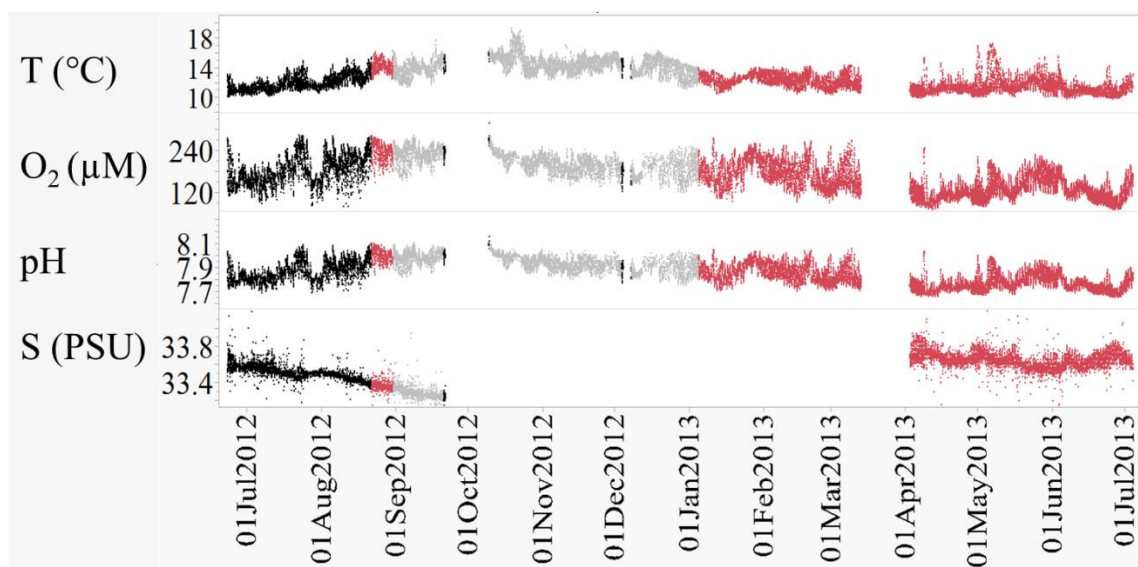


Figure 2.4. Seafloor environmental measurements (Sea pHox) at the McGowan site (32.86 °N, 117.28°W). Depth=30 m. Black = *D. opalescens* absent, Grey = *D. opalescens* not surveyed, Red = *D. opalescens* present.

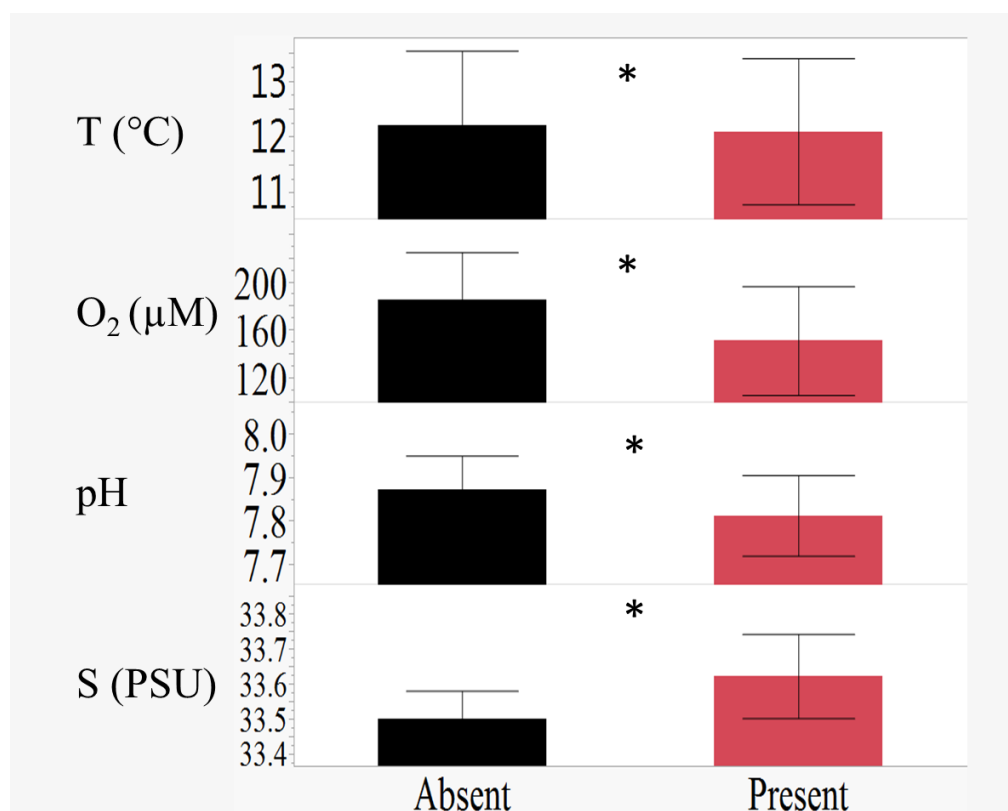


Figure 2.5. Seafloor environmental measurements (Sea pHox) and temperature loggers at the McGowan site (32.86 °N, 117.28°W). Depth = 25 - 30 m. Black = *D. opalescens* absent, Red = *D. opalescens* present. * = significant difference ($P < 0.05$; Wilcoxon test).

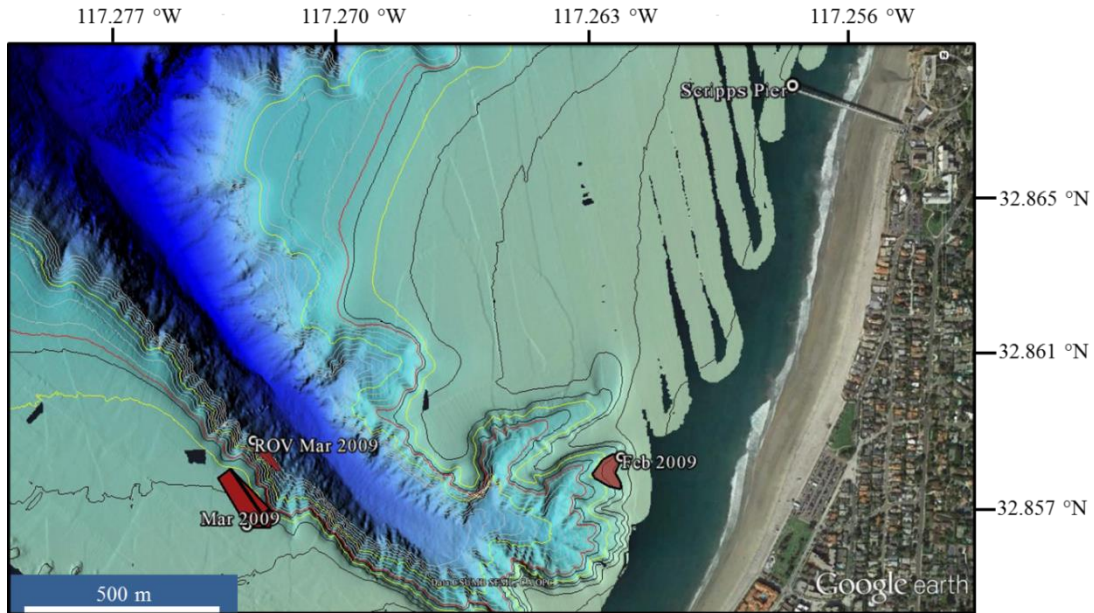


Figure 2.6. Recreational diver reports in February and March, 2009. Feb 2009 = Area where divers reported *D. opalescens* spawning are indicated by translucent red. Mar 2009 = Area where recreational divers observed and estimated *D. opalescens* embryo bed indicated by opaque red. Observations were recorded between 6 and 26 March, 2009. ROV Mar 2009 = Estimated area where embryos were observed indicated by translucent red. Observations were recorded between 72 and 98 m on 26 March, 2009 (Hawkins et al. 2010). Red contour = 40 m depth (Dive Limit for most Scientific Divers). Grey contours = 6.1 m increments of depth. Yellow contour = 30 m and 60 m depth increment.

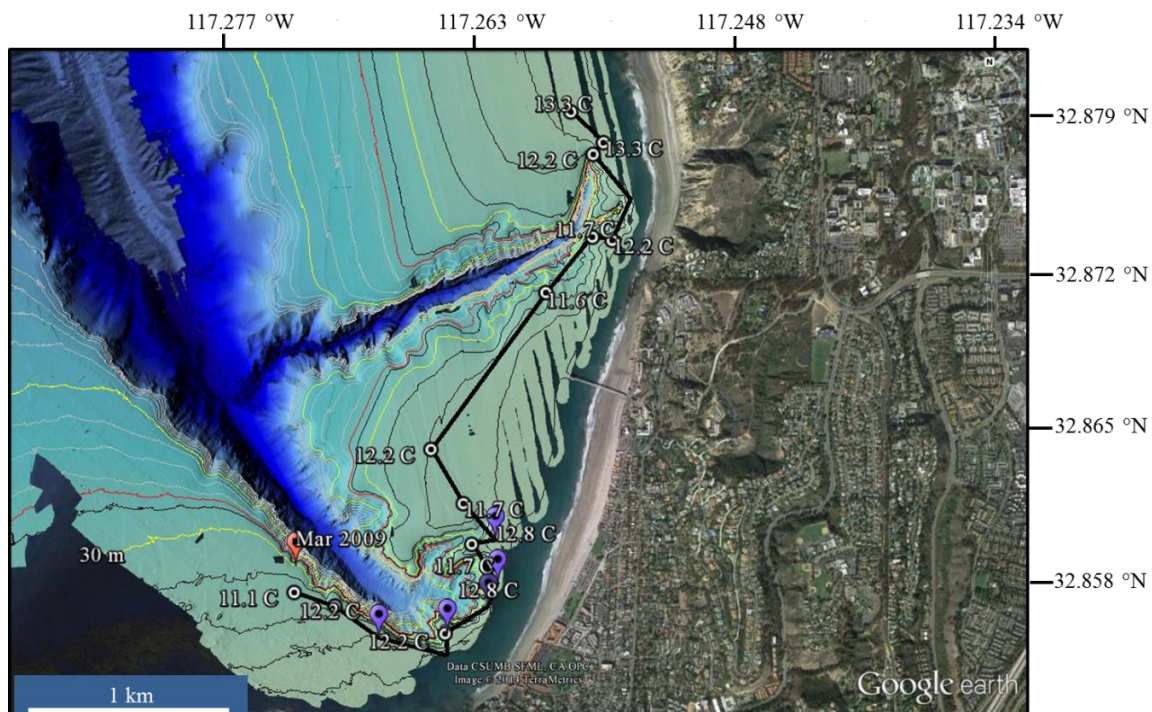


Figure 2.7. Absent squid-embryo beds or capsules were observed during a squid-embryo bed survey, 15 March, 2010. A 4.7 km transect survey was conducted using a drop-down camera within the 15-30 m depth contours at the McGowan site. Numbers = temperature at stations of the survey. Purple = *Dendraster excentricus* bed. Red = Squid embryo bed location in March, 2009. Temperature loggers were deployed here from 19 August, 2009 – 16 January, 2010. Yellow contour = 30 m and 60 m depth increment (30 m = Tether limit for the drop-down camera). Red contour = 40 m depth (Dive limit for most scientific divers). Grey contours = 6.1 m depth increments.

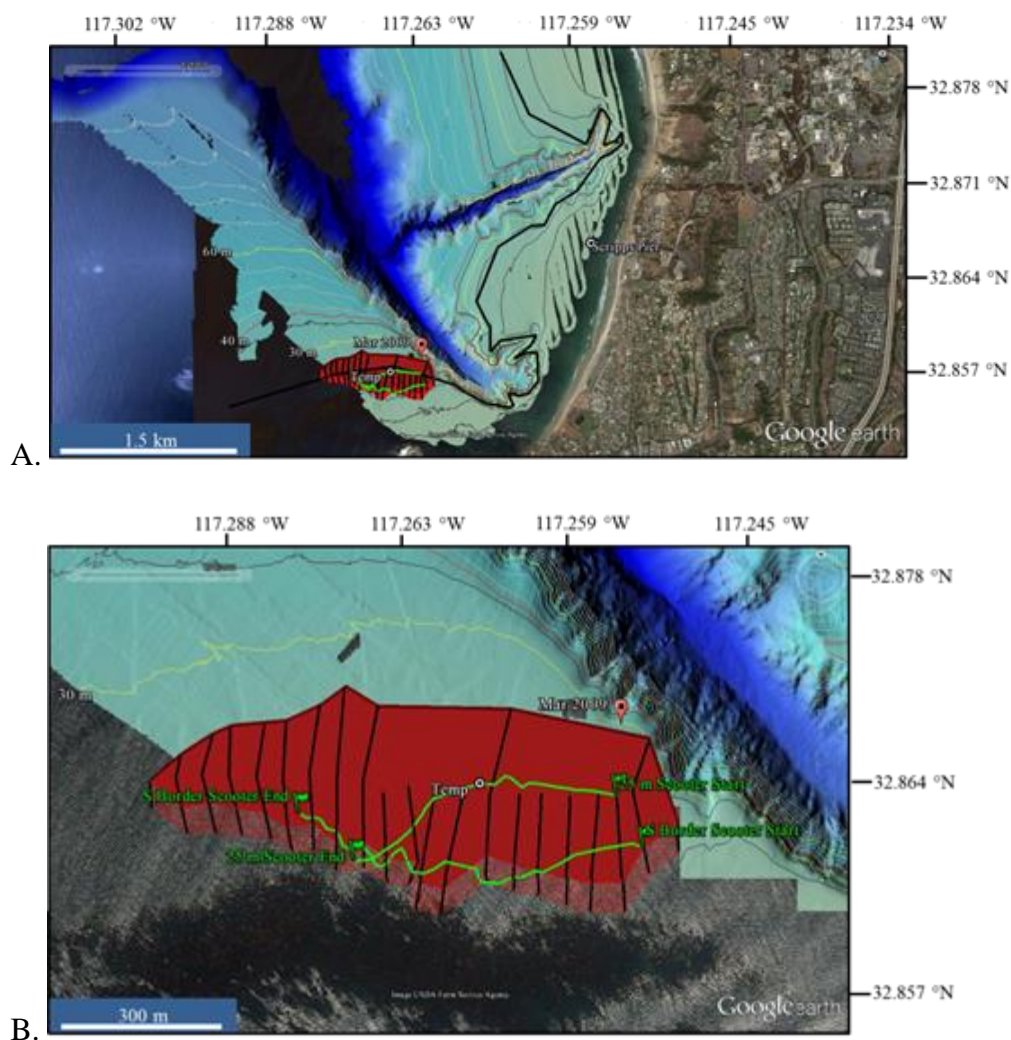


Figure 2.8. A. Squid-embryo beds were observed (red) at the McGowan site on 25 June, 2010 using a drop-down camera along the 25-30 m depth contours from 26 May – 8 June, 2010. B. The squid-embryo bed covered $2.3 \cdot 10^6 \text{ m}^2$ of the seafloor. Squid-embryo capsules did not occur (translucent red) within 50-m of the *Macrocystis pyrifera* (dark brown). Cross-bed transects (black lines) were conducted from 25 Jun– 25 Jul, 2010. An additional, transects (green) were conducted on 8 Jul, 2010 by SCUBA-divers using scooters. Temp = Temperature loggers deployed from 19 August, 2009 – 16 January, 2010. Yellow contour = 30 m and 60 m depth increment (30 m = Tether limit for the drop-down camera). Red contour = 40 m depth (Dive limit for scientific-transect surveys). Grey contours = 6.1 m depth increments.

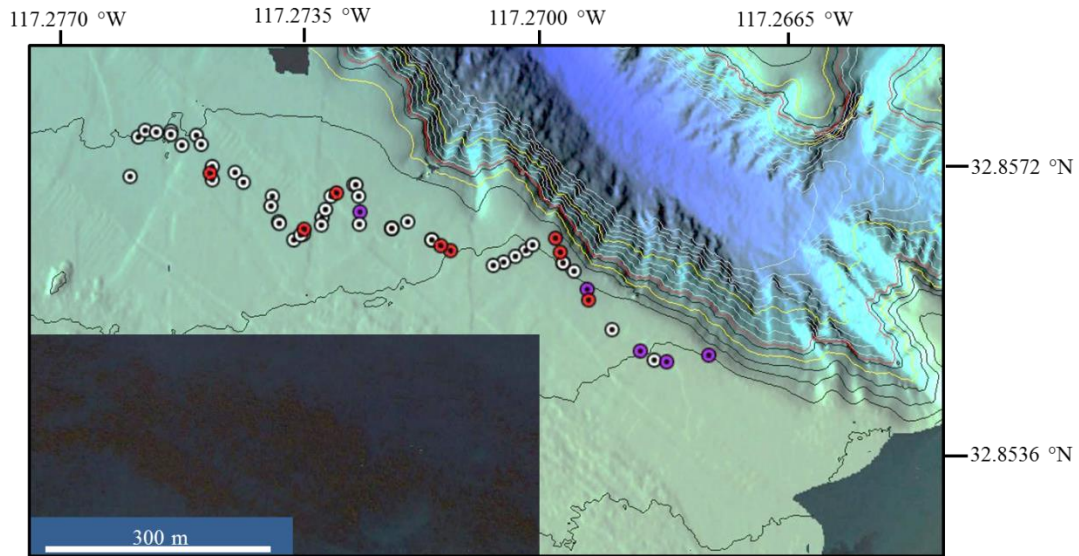


Figure 2.9. Squid-embryo beds were observed (red) at the McGowan site on 13 Jan, 2012 using a drop-down camera along the 25-30 m depth contours. *Macrocystis pyrifera* (dark brown) can be seen in the bottom left. Purple = *Dendraster excentricus* bed. Yellow contour = 30 m and 60 m depth increment (30 m = Tether limit for the drop-down camera). Red contour = 40 m depth. Grey contours = 6.1 m depth increments.

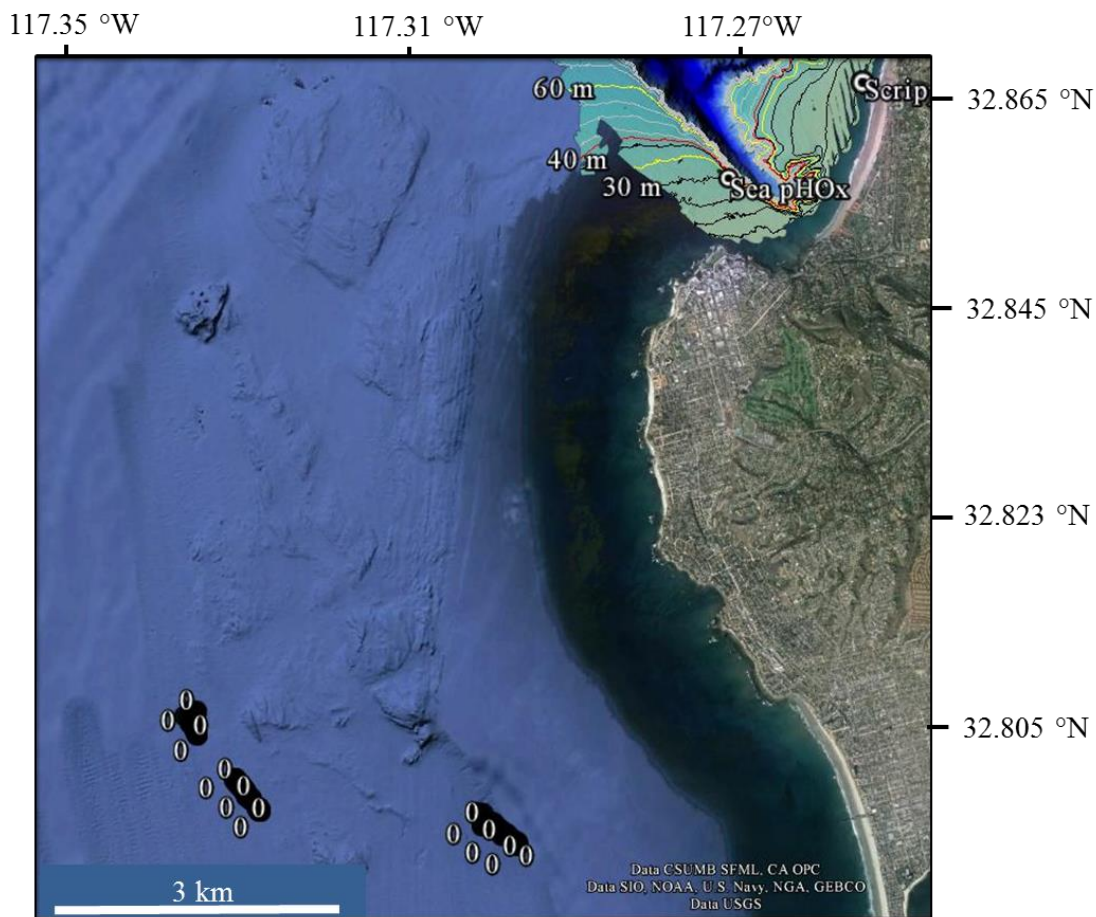


Figure 2.10. No squid-embryo beds were observed off the coast of Pacific Beach, 40-75 m depth, on 1 August, 2012. Three transects were conducted using the Honu ROV. SeapHOx = Sea pHOx Instrument deployed at the McGowan site from 23 June-21 September, 2012.

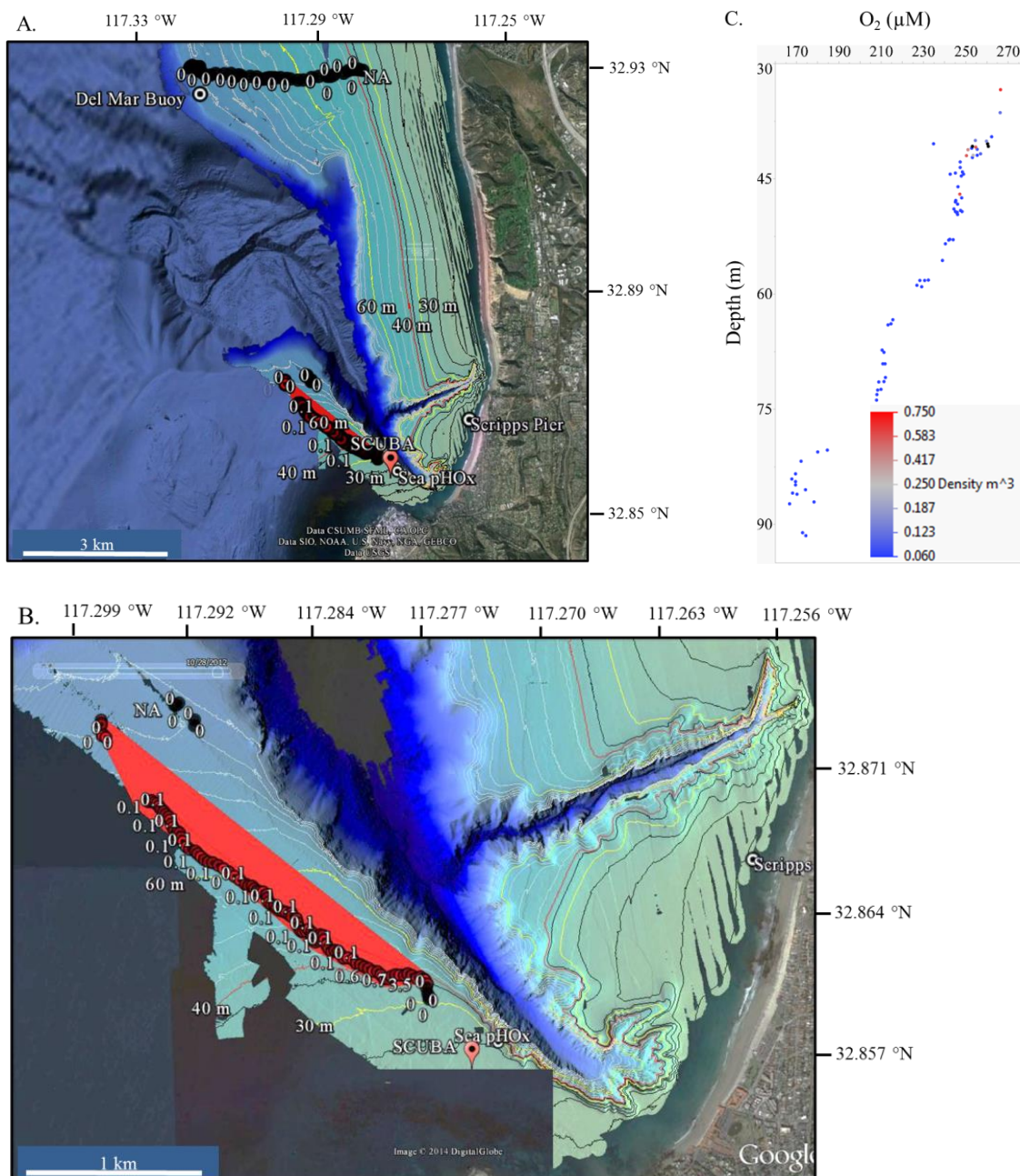


Figure 2.11. A. On 30 August, 2012 two surveys were conducted using the Honu ROV; one at Del Mar and the other at the McGowan Site. Squid-embryo beds were only observed (red) at the McGowan site. B. Squid-embryo bed at the McGowan site. Numbers next to points = Density of squid-embryo capsules \cdot m⁻². Kelp can be seen in the lower left portion of the graphic. SeapHOx = Sea pHox Instrument was deployed from 23 June-21 September, 2012. Yellow contour = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. C. O₂-depth profile with *D. opalescens*-embryo capsule density color-coded.

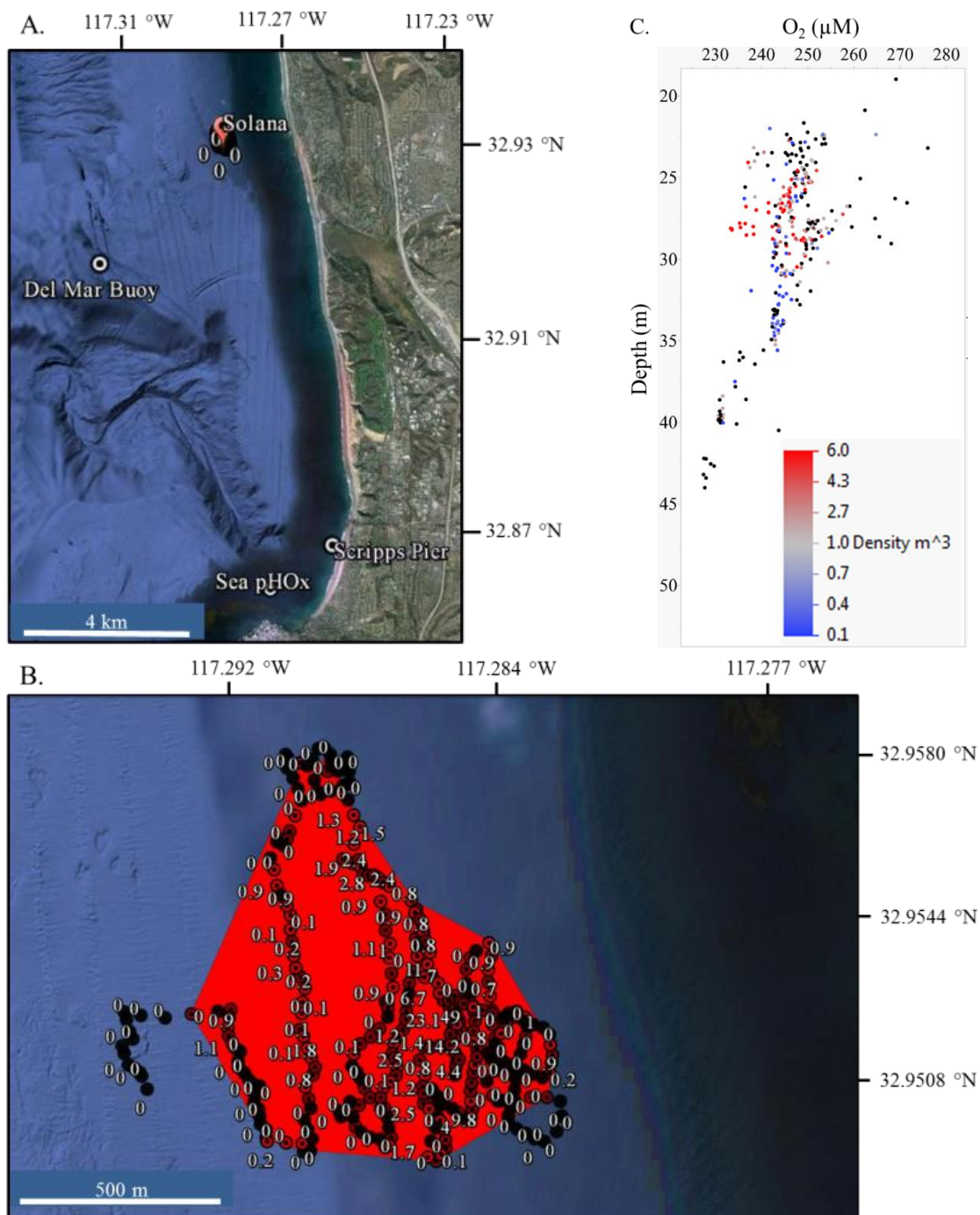


Figure 2.12. Squid-embryo beds were observed (red) at Solana Beach, USA on 30 September, 2012. A 2.5 km transect survey was conducted using the Honu ROV. B. Squid-embryo bed at Solana Beach. Numbers next to points = Density of squid-embryo capsules $\cdot \text{m}^{-2}$. C. O_2 -depth profile with *D. opalescens*-embryo capsule density color-coded.

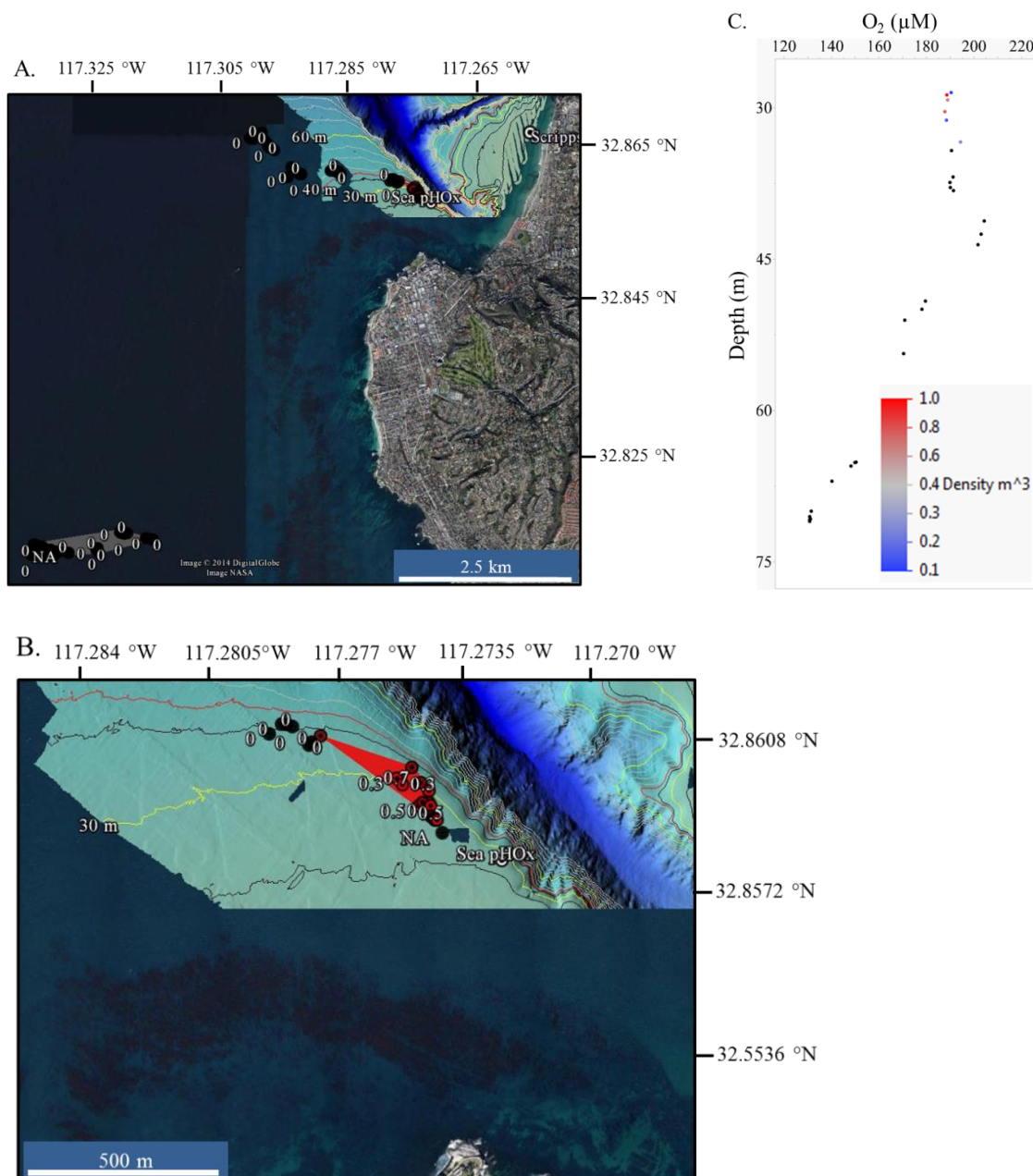


Figure 2.13. A. On 5 January, 2013 two surveys were conducted using the Honu ROV; Squid-embryo beds were only observed (red) at the McGowan site and not at Pacific Beach. B. Squid-embryo bed at the McGowan site. Numbers next to points = Density of squid-embryo capsules $\cdot m^{-2}$. SeapHOx = Sea pH/Ox Instrument was deployed from 7 December, 2012-13 March, 2013. Kelp can be seen in the lower portion of the graphic. Yellow contour = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. C. O_2 -depth profile with *D. opalescens*-embryo capsule density color-coded.

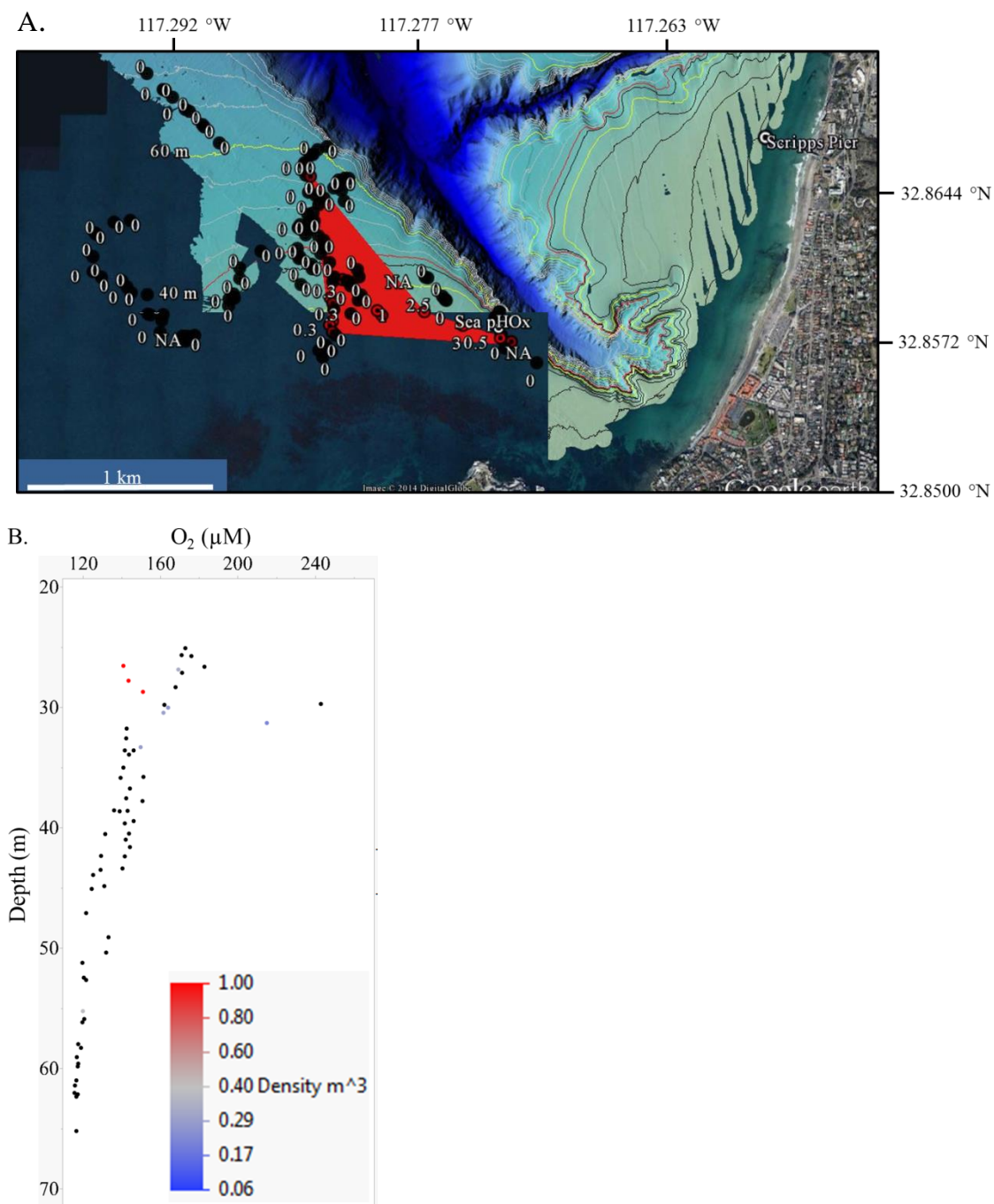


Figure 2.14. Squid-embryo beds were observed (red) at the McGowan site on 12 Feb, 2013 using the Honu ROV. Numbers next to points = Density of squid-embryo capsules $\cdot m^{-2}$. Kelp can be seen in the lower left corner of the graphic. SeapHOx = Sea pH/Ox Instrument deployed from 7 December, 2012- 13 March, 2013. Yellow contour = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. B. O_2 -depth profile with *D. opalescens*-embryo capsule density color-coded.

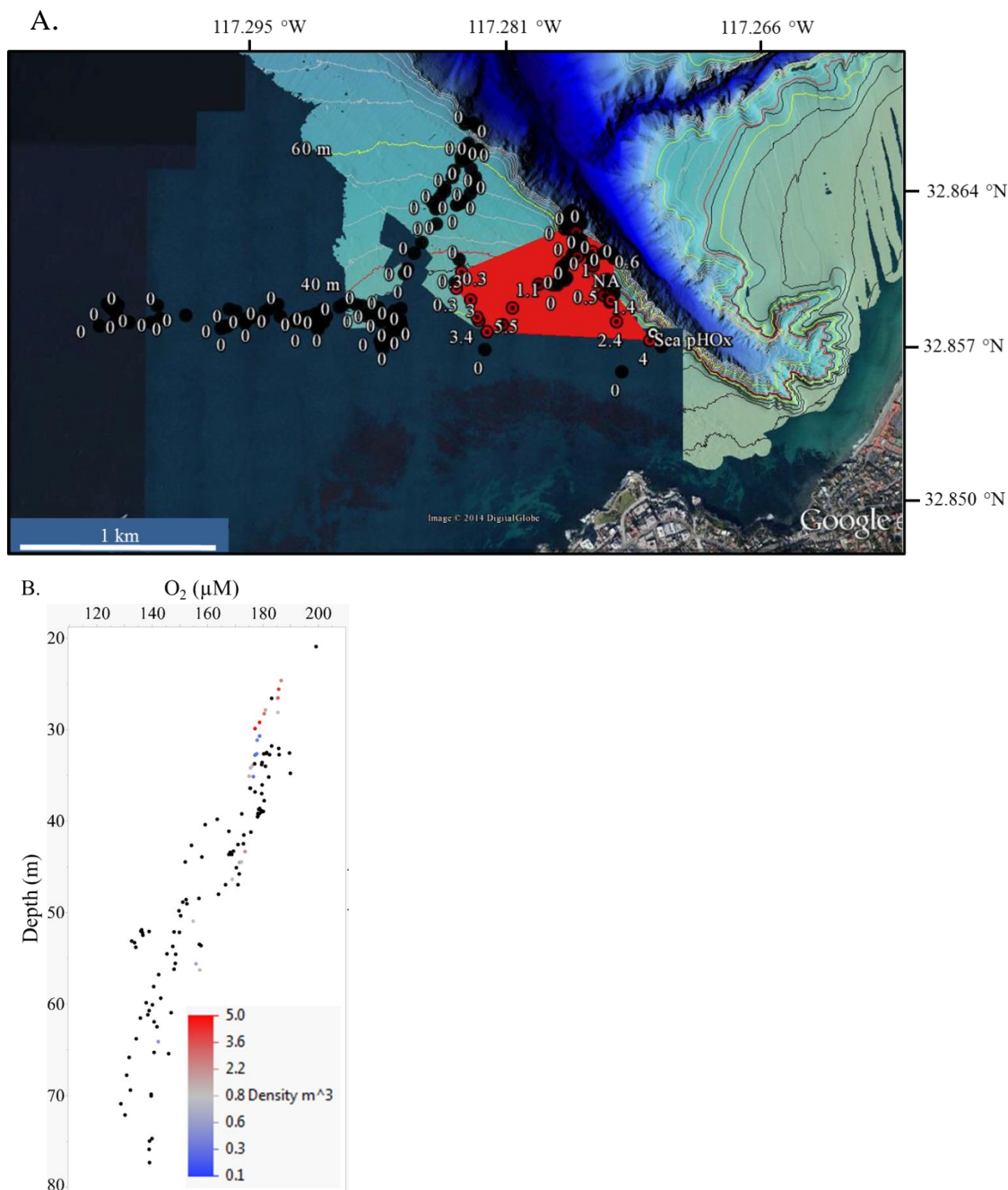


Figure 2.15. A. Squid-embryo beds were observed (red) at the McGowan site on 5 March, 2013 using the Honu ROV. Numbers next to points = Density of squid-embryo (capsules \cdot m⁻²). Kelp can be seen in the lower left corner of the graphic. SeapHOx = Sea pHOx was deployed from 7 December, 2012-13 March, 2013. Yellow contour = 30 m and 60 m depth increment Red contour = 40 m depth. Grey contours = 6.1 m depth increments. B. O₂-depth profile with *D. opalescens*-embryo capsule density color-coded.

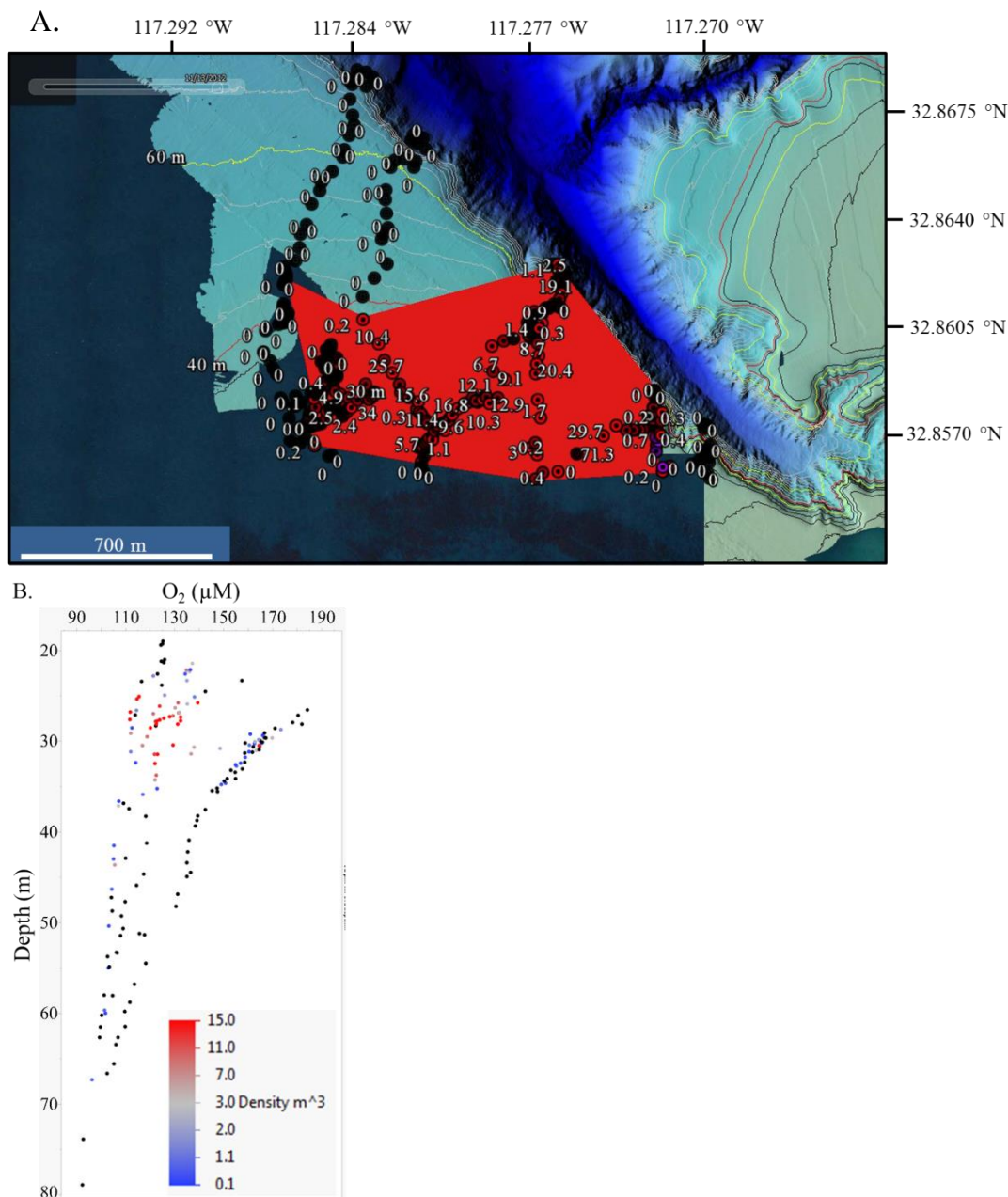


Figure 2.16. Squid-embryo beds were observed (red) at the McGowan site on 26 March, 2013 using the ROV Honu. The squid-embryo bed covered $7.3 \cdot 10^6 \text{ m}^2$ of the seafloor. Numbers next to points = Density of squid-embryo (capsules $\cdot \text{m}^{-2}$). Kelp can be seen in the lower left corner of the graphic. SeapHOx = Sea pHOX was deployed from 7 December, 2012- 13 March, 2013 and from 2 April, 2013 - 4 July, 2013. Yellow contour = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. B. O_2 -depth profile with *D. opalescens*-embryo capsule density color-coded.

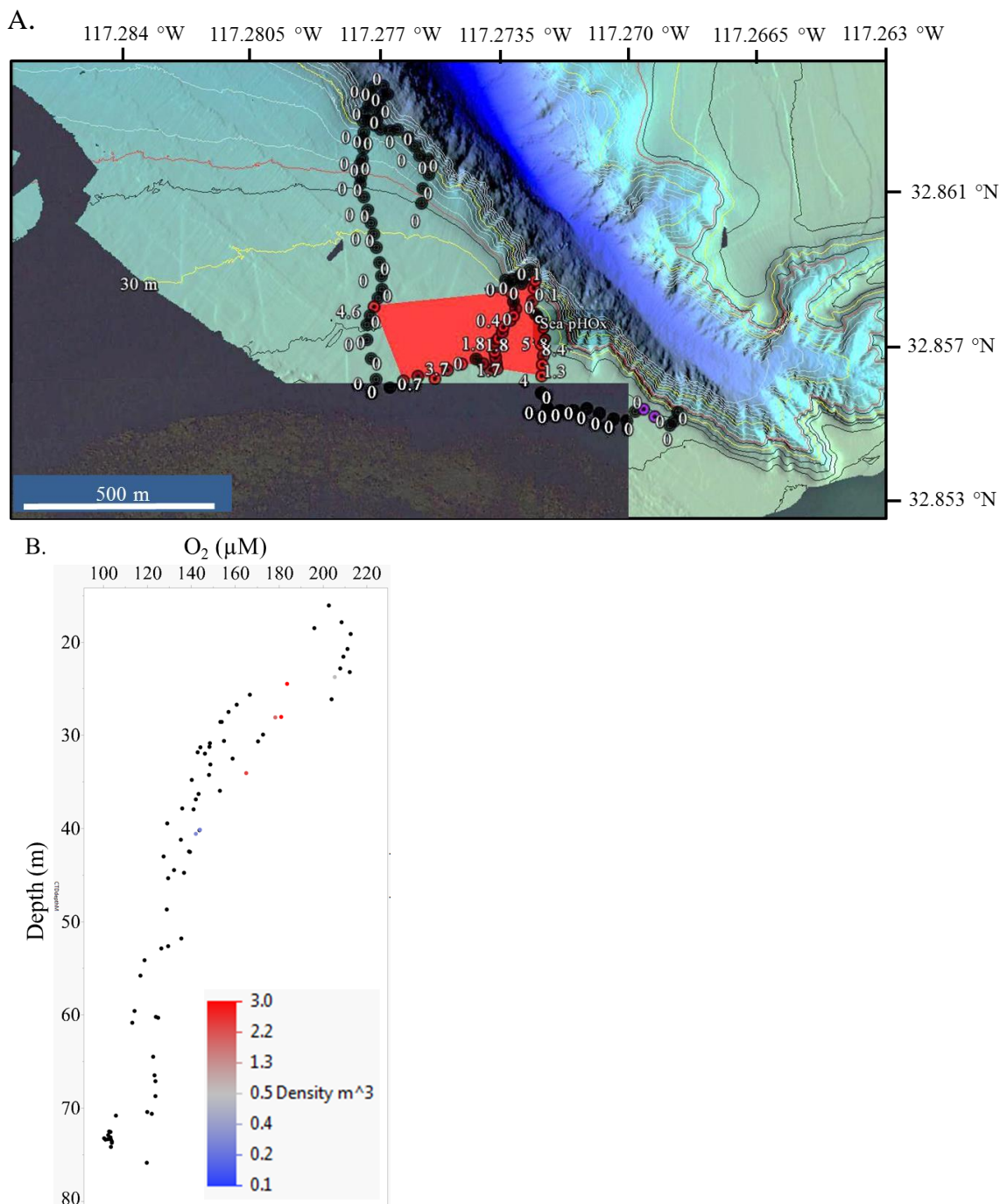


Figure 2.17. A. Squid-embryo beds were observed (red) at the McGowan site on 14 June, 2013 using the ROV Honu. Numbers next to points = Density of squid-embryo (capsules $\cdot\text{m}^{-2}$). Kelp can be seen in the lower left corner of the graphic. Purple points = *Dendraster excentricus*. SeapHOx = Sea pHOx was deployed from 2 April, 2013 - 4 July, 2013. Yellow contour = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. B. O_2 -depth profile with *D. opalescens*-embryo capsule density color-coded.

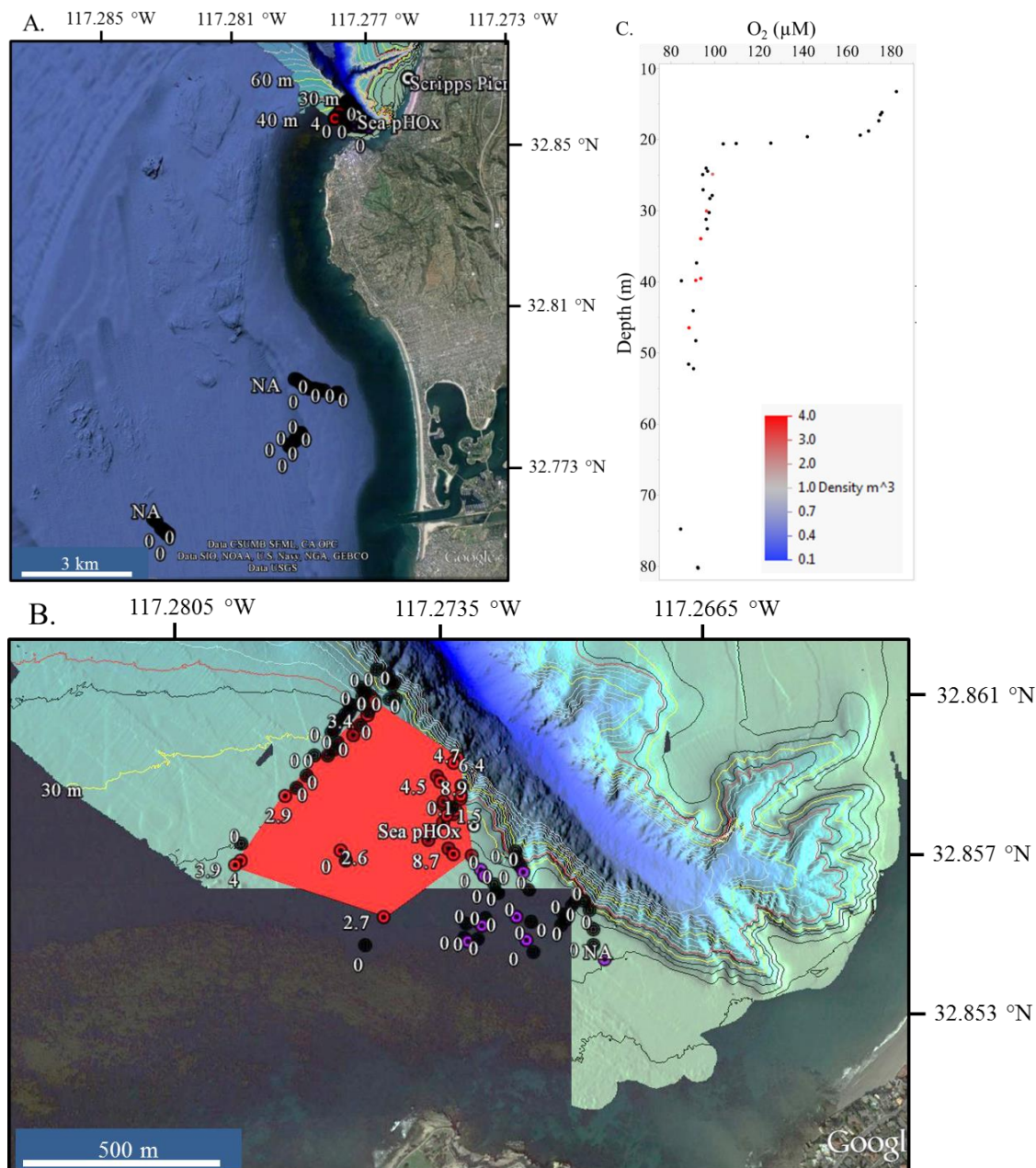


Figure 2.18. A. On 26 June, 2013 two surveys were conducted using the Honu ROV; one at the McGowan site and the other at Pacific Beach. Squid-embryo beds were only observed (red) at the McGowan site. B. Zoom-in of the the squid-embryo bed that covered $1.9 \cdot 10^6 \text{ m}^2$ of the seafloor. SeapHOx = Sea pHox Instrument was deployed from 7 December, 2012-13 March, 2013. Numbers next to points = Density of squid-embryo capsules $\cdot \text{m}^{-2}$. Kelp can be seen in the lower portion of the graphic. Yellow contour = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. C. O₂-depth profile with *D. opalescens*-embryo capsule density color-coded.

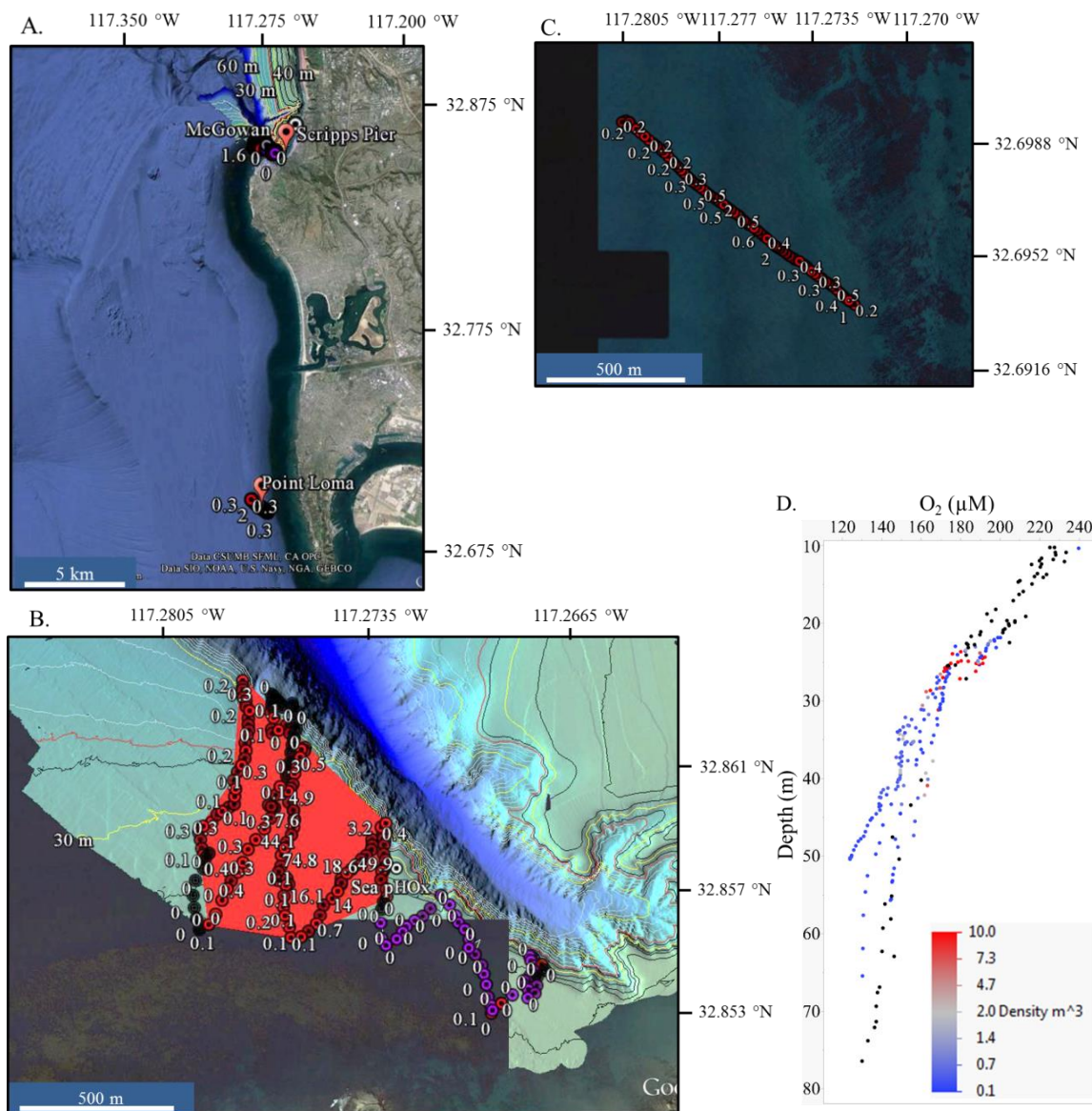


Figure 2.19. A. Squid-embryo beds were observed (red) at the McGowan site and at Point Loma on 30 July, 2013 using the ROV Honu. B. The squid-embryo bed covered at the McGowan site. Numbers next to points = Density of squid-embryo (capsules·m⁻²). Kelp can be seen in the lower left corner of the graphic. Purple points = *Dendraster excentricus*. SeapHOx = Sea pHOX was deployed from 2 April, 2013-4 July, 2013. Yellow contours = 30 m and 60 m depth increment. Red contour = 40 m depth. Grey contours = 6.1 m depth increments. Numbers next to points = Density of squid-embryo (capsules·m⁻²). C. Squid embryo observations at Point Loma, USA. Numbers next to points = Density of squid-embryo (capsules·m⁻²). D. O₂-depth profile with *D. opalescens*-embryo capsule density color-coded.

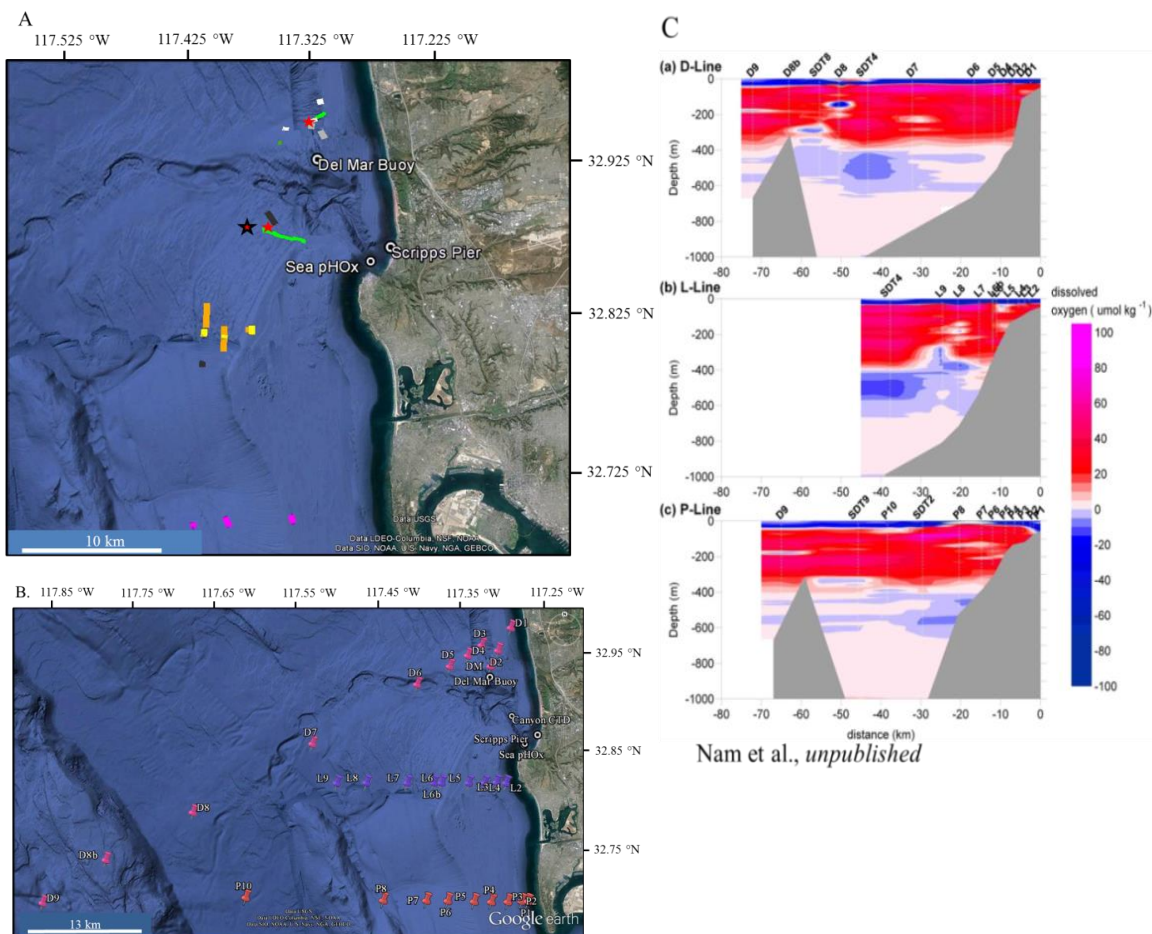


Figure 2.20. R/V Melville San Diego Coastal Expedition A. Seafloor surveys: No spawning/mating *D. opalescens* or *D. opalescens* embryo capsules were observed during ROV Triton surveys (green lines) on 3 July, 7 and 8 Dec, 2012. No *D. opalescens*, at any life stage, were captured in otter trawls at 100 (N=5), 300 (N=5), or at 400 m depth (N=6; Orange and grey lines = July 3-9, 2012; white, yellow and pink line = December 8-14, 2012). Red star = Adult, non-spawning squid B. CTD survey stations. C. Courtesy of SungHyun Nam: Oxygen changes over the survey area between July and December, 2012 on the (a) D-line, (b) L-line and (c) P-line (Adapted from Nam et al. unpublished).

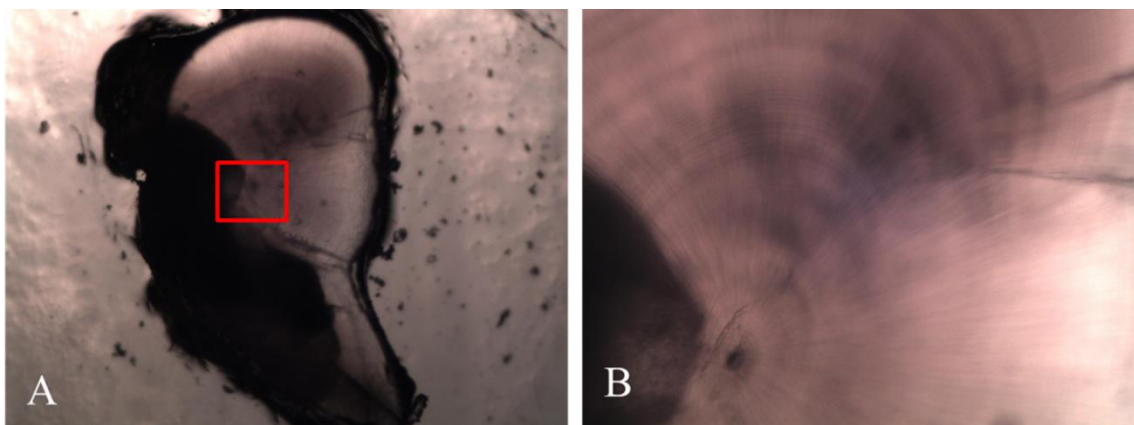


Figure 2.21. A polished statolith from a post-spawn *D. opalescens* (cohort = 11 November, 2010). Images were taken using a compound microscope. A. Statolith. Red square = natal core. B. Natal core. Each pair of dark and light “rings” indicates one day (Yang et al. 1986, Jackson 1994a).

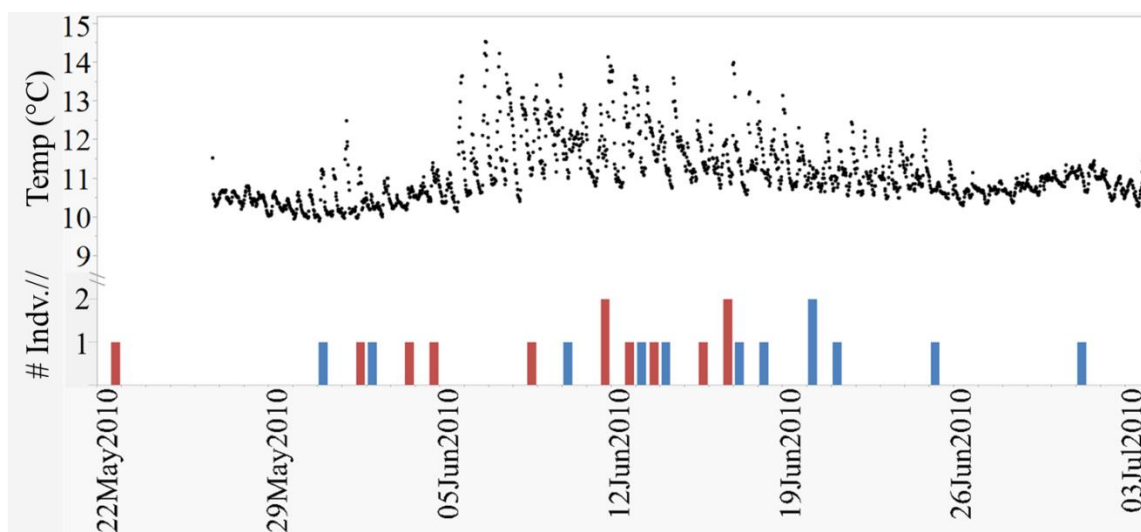


Figure 2.22. McGowan site seafloor temperature (°C; 25 m depth) and back-calculated number of individuals from post-spawned squid collected at the McGowan site on 28 Oct (blue) or 11 Nov, 2010 (red). Although this graphic overlays the birth date of the squid with the temperature at the McGowan site, there is no certainty that post-spawned squid collected at the McGowan site developed here as embryos.

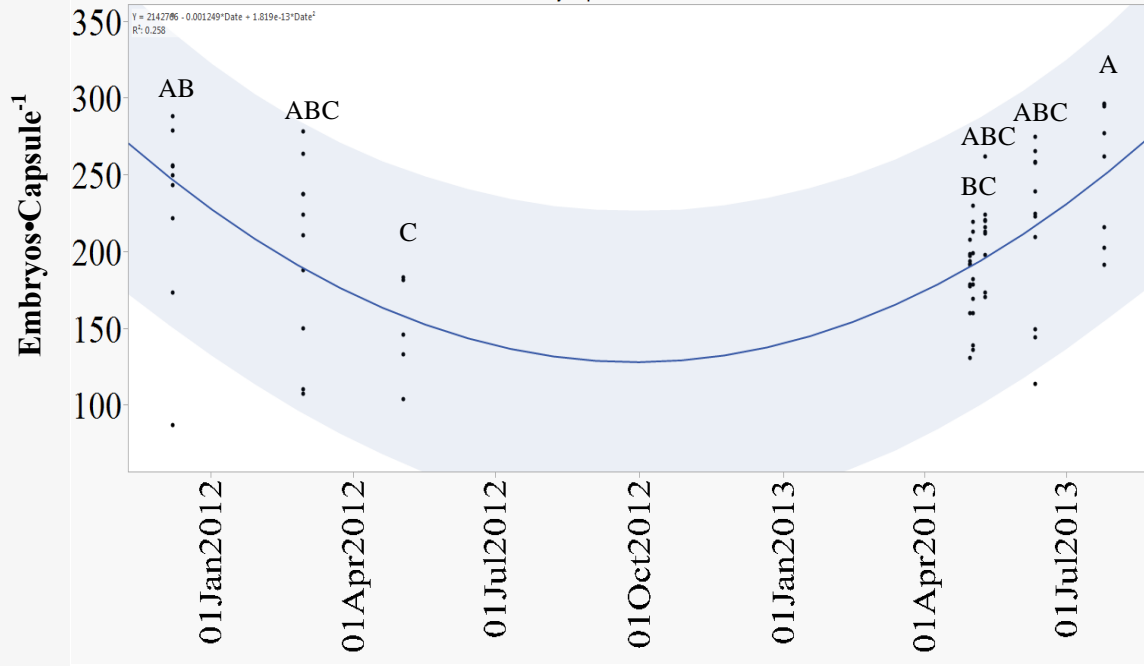


Figure 2.23. A. Squid-embryo•capsule⁻¹ counts for collections on 8 Dec, 2012; 29 Feb, 3 May, 2012; 30 Apr, 2 May, 10 May, 11 Jun, 25 July, 2013 at the McGowan site. Quadratic relationship ($R=0.26$) between the count and date were used to estimate embryo•capsule⁻¹ for field surveys. Letters indicate distinction among groups.

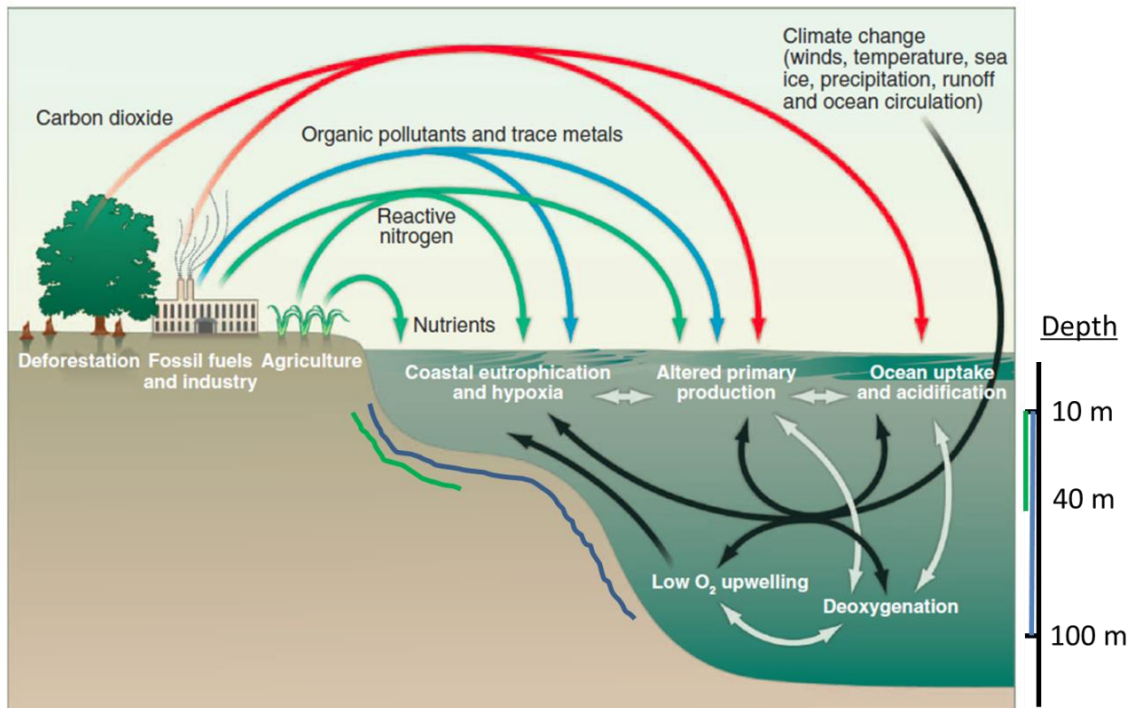


Figure 2.24. Adaptation from Doney et al. 2010 to show a hypothetical mechanism for the distribution embryo habitat based on the environmental conditions on the shelf. In this scenario, *D. opalescens* spawning adults select sites according to oceanographic conditions that benefit their offspring (embryos). When temperature, [O₂], and pH are low (*p*CO₂ is high) on the shelf (i.e. green line), spawners compress to the upper shelf. Alternatively, when temperature, [O₂] and pH are high (*p*CO₂ is high), spawners utilize the entire shelf.

Tables

Table 2.1. Instrument, location, depth and date (s) of deployment.

Instrument	Coordinates	Location	Depth	Deployment Date (s)
CTD with O ₂ Optode	32.89 °N, 117.29 °W	Submarine Canyon	350 m	18 Aug, 2009
Temperature Loggers	32.86 °N, 117.28 °W	McGowan Site	25 m	18 Aug-22 Sep, 2009
	32.86 °N, 117.28 °W	McGowan Site	25 m	23 Sep-9 Dec, 2009
	32.86 °N, 117.28 °W	McGowan Site		5 Dec, 2009-23 Jan, 2010
	32.86 °N, 117.28 °W	McGowan Site		5 Feb, 2010-27 Mar, 2010
	32.86 °N, 117.28 °W	McGowan Site		26 May-3 Nov, 2010
Sea pH _{Ox}	32.86 °N, 117.28 °W	McGowan Site	30 m	16 Jul, 2010-26 Jan, 2011
CTD with O ₂ Optode and discrete carbonate measurements	32.97 °N, 117.29 °W (NE station)	San Diego Region (28 stations; Figure 2.20.)	0-1000 m	30 June-10 Jul, 2012
	32.69 °N, 117.86 °W (SW station)			
	32.69 °N, 117.27 °W (SE station)			
Sea pH _{Ox}	32.86 °N, 117.28 °W	McGowan Site	30 m	9 Oct-4 Dec, 2012; 7 Dec, 2012-13 Mar, 2013
CTD with O ₂ Optode and discrete carbonate measurements	32.97 °N, 117.29 °W (NE station)	San Diego Region (28 stations; Figure 2.20.)	0-1000 m	8-15 Dec, 2012
	32.69 °N, 117.86 °W (SW station)			
	32.69 °N, 117.27 °W (SE station)			
Sea pH _{Ox}	32.86 °N, 117.28 °W	McGowan Site	30 m	2 Apr-4 Jul, 2013

Table 2.2. Spatial Surveys: Environmental factors in and out of *D. opalescens* beds from ROV Honu surveys (8/1/2012 – 7/30/2013; depth range = 11.2 - 94.5 m). pH was estimated using temperature and oxygen data (Alin et al. 2012). Black = Data acquired when *D. opalescens* embryo capsules were absent, Red = Data acquired when *D. opalescens* embryo capsules were present. AVG = Average, STDEV = Standard deviation, MAX = Maximum, MIN = Minimum, N = # of samples. Bold and italicized = significant.

Stat.	T(°C)	T(°C)	[O ₂](μM)	[O₂](μM)	pH _{est}	pH_{est}	D(m)	D(m)	S (PSU)	S (PSU)
AVG	11.63	12.49	161.6	186.7	7.818	7.876	45.5	35.6	33.57	33.51
STDEV	1.45	1.71	47.1	52.2	0.103	0.116	19.7	13.1	0.12	0.12
MAX	16.45	15.46	276.9	266.3	8.110	8.060	94.4	91.5	33.93	33.90
MIN	9.59	9.87	84.4	88.2	7.660	7.670	11.2	11.3	33.35	33.31
N	1101	713	1101	713	1101	713	1101	713	1101	713
χ^2	118.6		93.4		104.4		120.2		114.2	
<i>P</i>	< 0.0001		< 0.0001		< 0.0001		< 0.0001		< 0.0001	

Table 2.3. Temporal Survey: Environmental conditions of the squid embryo habitat at the McGowan site over time at 30 m depth. Temperature data are from the Hobo Tidbit® Loggers (8/14/2009 – 1/28/2011) and the SeapHOx instrument (6/24/2012 – 7/4/2013). [O₂], pH and S are all from the SeapHOx instrument (6/24/2012 – 7/4/2013). Black = Data acquired when *D. opalescens* embryo capsules were absent, Red = Data acquired when *D. opalescens* embryo capsules were present. AVG = Average, STDEV = Standard deviation, MAX = Maximum, MIN = Minimum, N = # of samples. Bold and italicized = significant.

Stat.	T (°C)	T (°C)	[O ₂] (μM)	[O ₂] (μM)	pH	pH	S (PSU)	S (PSU)
AVG	12.21	12.09	185.3	150.9	7.870	7.812	33.50	33.62
STDEV	1.33	1.31	39.3	45.4	0.079	0.093	0.79	0.12
MAX	20.9	18.1	317.1	280.3	8.160	8.100	33.75	33.91
MIN	9.88	9.77	79.0	70.1	7.690	7.650	33.22	33.30
N	8,659	26,741	5,893	15,310	5,893	15,310	5816	9784
χ^2	57.8		2,528.1		1,957.2		4,916.8	
<i>P</i>	< 0.0001		< 0.0001		< 0.0001		< 0.0001	

Table 2.4. Tow-camera surveys.

#	Date	Coordinates	Location	<i>D. opalescens</i> embryos	Depth Range
1	15 Mar, 2010	32.86 °N, 117.28 °W	McGowan Site	Absent	20-30 m
2	26 May- 15 Jun, 2010	32.86 °N, 117.28 °W	McGowan Site	Absent	20-30 m
3	23 Jun- 30 Jul, 2010	32.86 °N, 117.28 °W	McGowan Site	Present	20-30 m
4	7-21 Oct, 2010	32.86 °N, 117.28 °W	McGowan Site	Absent	20-30 m
5	28 Oct-2 Nov, 2010	32.86 °N, 117.28 °W	McGowan Site	Present	20-30 m
6	14 Jan-15 Feb, 2011	32.86 °N, 117.28 °W	McGowan Site	Present	20-30 m
7	4 May, 2011	32.86 °N, 117.28 °W	McGowan Site	Absent	20-30 m
8	13 Jan, 2012	32.86 °N, 117.28 °W	McGowan Site	Present	20-30 m

Table 2.5. SCUBA Surveys.

#	Date	Coordinates	Site	<i>D. opalescens</i> embryos	Depth(m)	Temp(C)
1	27-Feb-2009	32.86 °N, 117.28°W	McGowan	Absent	24.7	12.2
2	29-Mar-2009	32.86 °N, 117.28°W	McGowan	Present	25.6	12.2
3	29-May-2009	32.86 °N, 117.28°W	McGowan	Present	28.7	12.8
4	18-Jul-2009	32.86 °N, 117.28°W	McGowan	Absent	27.7	15.0
5	18-Aug-2009	32.86 °N, 117.28°W	McGowan	Absent	24.7	13.9
6	23-Sep-2009	32.86 °N, 117.28°W	McGowan	Absent	31.4	17.2
7	23-Sep-2009	32.86 °N, 117.28°W	McGowan	Absent	14.6	15.0
8	26-Sep-2009	32.86 °N, 117.28°W	McGowan	Absent	24.4	13.9
9	29-Sep-2009	32.86 °N, 117.28°W	McGowan	Absent	28.4	13.9
10	5-Dec-2009	32.86 °N, 117.28°W	McGowan	Absent	32.3	13.9
11	5-Dec-2009	32.86 °N, 117.28°W	McGowan	Absent	15.5	13.9
12	5-Dec-2009	32.86 °N, 117.28°W	McGowan	Absent	25.3	15.0
13	5-Feb-2010	32.86 °N, 117.28°W	McGowan	Present	24.1	12.8
14	23-Jun-2010	32.86 °N, 117.28°W	McGowan	Present	24.1	11.1
15	23-Jun-2010	32.86 °N, 117.28°W	McGowan	Present	24.7	11.1
16	8-Jul-2010	32.86 °N, 117.28°W	McGowan	Present	23.8	12.2
17	8-Jul-2010	32.86 °N, 117.28°W	McGowan	Present	24.1	10.0
18	16-Jul-2010	32.86 °N, 117.28°W	McGowan	Present	25.3	10.0
19	16-Jul-2010	32.86 °N, 117.28°W	McGowan	Present	25.9	10.0
20	19-Aug-2010	32.86 °N, 117.28°W	McGowan	Present	25.6	10.0

Table 2.5. SCUBA Surveys (cont.).

#	Date	Coordinates	Site	<i>D. opalescens</i> embryos	Depth(m)	Temp(C)
21	19-Aug-2010	32.86 °N, 117.28°W	McGowan	Present	25.6	10.0
22	26-Aug-2010	32.86 °N, 117.28°W	McGowan	Present	26.2	10.0
23	14-Sep-2010	32.86 °N, 117.28°W	McGowan	Present	19.2	10.0
24	15-Sep-2010	32.86 °N, 117.28°W	McGowan	Present	25.9	11.1
25	15-Sep-2010	32.86 °N, 117.28°W	McGowan	Absent	22.6	11.1
26	22-Sep-2010	32.86 °N, 117.28°W	McGowan	Present	25.0	11.1
27	26-Sep-2010	32.86 °N, 117.28°W	McGowan	Present	18.3	13.9
28	29-Oct-2010	32.86 °N, 117.28°W	McGowan	Present	26.8	13.9
29	11-Nov-2010	32.86 °N, 117.28°W	McGowan	Present	25.9	12.2
30	11-Nov-2010	32.86 °N, 117.28°W	McGowan	Present	19.8	12.8
31	15-Nov-2010	32.86 °N, 117.28°W	McGowan	Absent	30.5	12.2
32	15-Nov-2010	32.86 °N, 117.28°W	McGowan	Absent	30.5	16.1
33	17-Nov-2010	32.86 °N, 117.28°W	McGowan	Present	25.9	12.8
34	18-Nov-2010	32.86 °N, 117.28°W	McGowan	Present	18.3	13.9
35	18-Nov-2010	32.86 °N, 117.28°W	McGowan	Present	18.3	13.9
36	28-Dec-2010	32.86 °N, 117.28°W	McGowan	Present	13.7	13.9
37	26-Jan-2011	32.86 °N, 117.28°W	McGowan	Present	25.6	11.1
38	26-Jan-2011	32.86 °N, 117.28°W	McGowan	Present	18.9	11.1
39	7-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	26.8	11.1
40	14-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	24.4	12.2
41	14-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	26.8	11.1
42	15-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	24.1	12.2
43	15-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	24.1	8.9
44	16-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	24.4	8.9
45	17-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	24.1	8.9
46	17-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	24.1	8.9
47	20-Feb-2011	32.86 °N, 117.28°W	McGowan	Present	23.8	8.9
48	23-Aug-2011	32.86 °N, 117.28°W	McGowan	Present	27.1	12.8
49	30-Aug-2011	32.86 °N, 117.28°W	McGowan	Present	29.6	11.1
50	22-Sep-2011	32.86 °N, 117.28°W	McGowan	Present	29.9	12.2
51	28-Oct-2011	32.86 °N, 117.28°W	McGowan	Absent	29.6	10.0
52	11-Nov-2011	32.86 °N, 117.28°W	McGowan	Absent	18.0	12.8
53	11-Nov-2011	32.86 °N, 117.28°W	McGowan	Absent	16.8	11.1
54	7-Dec-2011	32.86 °N, 117.28°W	McGowan	Present	26.2	12.8

Table 2.5. SCUBA Surveys (cont.).

#	Date	Coordinates	Site	<i>D. opalescens</i> embryos	Depth(m)	Temp(C)
55	7-Dec-2011	32.86 °N, 117.28°W	McGowan	Present	19.2	12.8
56	8-Dec-2011	32.86 °N, 117.28°W	McGowan	Present	13.4	12.8
57	8-Dec-2011	32.86 °N, 117.28°W	McGowan	Present	17.4	12.8
58	9-Dec-2011	32.86 °N, 117.28°W	McGowan	Present	26.8	15.0
59	31-Jan-2012	32.86 °N, 117.28°W	McGowan	Present	25.9	12.2
60	31-Jan-2012	32.86 °N, 117.28°W	McGowan	Present	18.6	13.3
61	29-Feb-2012	32.86 °N, 117.28°W	McGowan	Present	25.9	12.2
62	29-Feb-2012	32.86 °N, 117.28°W	McGowan	Absent	23.5	12.2
63	28-Mar-2012	32.88 °N, 117.26°W	Scripps	Present	18.9	12.2
64	30-Apr-2012	32.86 °N, 117.28°W	McGowan	Absent	16.8	16.1
65	3-May-2012	32.86 °N, 117.28°W	McGowan	Present	25.6	13.9
66	3-May-2012	32.86 °N, 117.28°W	McGowan	Present	25.0	12.2
67	4-May-2012	32.86 °N, 117.28°W	McGowan	Absent	18.6	13.9
68	4-May-2012	32.86 °N, 117.28°W	McGowan	Absent	18.6	17.2
69	19-Jun-2012	32.86 °N, 117.28°W	McGowan	Absent	30.5	12.2
70	22-Jun-2012	32.86 °N, 117.28°W	McGowan	Absent	30.5	11.1
71	27-Jun-2012	32.86 °N, 117.28°W	McGowan	Absent	30.5	11.1
72	27-Jun-2012	32.86 °N, 117.28°W	McGowan	Absent	30.5	11.1
73	21-Aug-2012	32.86 °N, 117.28°W	McGowan	Absent	19.2	12.8
74	22-Aug-2012	32.86 °N, 117.28°W	McGowan	Absent	31.1	17.2
75	22-Aug-2012	32.86 °N, 117.28°W	McGowan	Present	26.8	20.0
76	24-Aug-2012	32.86 °N, 117.28°W	McGowan	Absent	22.9	16.1
77	24-Aug-2012	32.86 °N, 117.28°W	McGowan	Present	19.5	20.0
78	21-Sep-2012	32.86 °N, 117.28°W	McGowan	Absent	31.1	12.2
79	21-Sep-2012	32.86 °N, 117.28°W	McGowan	Absent	19.2	13.9
80	9-Oct-2012	32.86 °N, 117.28°W	McGowan	Absent	32.0	16.1
81	4-Dec-2012	32.86 °N, 117.28°W	McGowan	Absent	31.1	12.8
82	7-Dec-2012	32.86 °N, 117.28°W	McGowan	Absent	30.5	12.2
83	13-Mar-2013	32.86 °N, 117.28°W	McGowan	Present	32.3	11.1
84	19-Mar-2013	32.86 °N, 117.28°W	McGowan	Present	28.0	11.1
85	1-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	30.5	10.0
86	2-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	32.3	10.0
87	3-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	29.3	10.0
88	5-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	28.4	10.0

Table 2.5. SCUBA Surveys (cont.).

#	Date	Coordinates	Site	<i>D. opalescens</i> embryos	Depth(m)	Temp(C)
89	5-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	24.1	8.9
90	10-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	29.9	8.9
91	24-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	30.2	8.9
92	26-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	30.8	10.0
93	30-Apr-2013	32.86 °N, 117.28°W	McGowan	Present	23.5	11.1
94	1-May-2013	32.86 °N, 117.28°W	McGowan	Present	39.0	12.8
95	2-May-2013	32.86 °N, 117.28°W	McGowan	Present	23.5	12.8
96	10-May-2013	32.86 °N, 117.28°W	McGowan	Present	25.0	11.1
97	4-Jun-2013	32.86 °N, 117.28°W	McGowan	Present	24.7	12.8
98	11-Jun-2013	32.86 °N, 117.28°W	McGowan	Present	39.9	8.9
99	2-Jul-2013	32.86 °N, 117.28°W	McGowan	Present	24.4	12.2
100	4-Jul-2013	32.86 °N, 117.28°W	McGowan	Present	29.9	12.2
101	4-Jul-2013	32.86 °N, 117.28°W	McGowan	Present	29.6	11.1
102	25-Jul-2013	32.86 °N, 117.28°W	McGowan	Present	29.6	10.0
103	25-Jul-2013	32.86 °N, 117.28°W	McGowan	Present	24.7	8.9
104	14-Aug-2013	32.86 °N, 117.28°W	McGowan	Present	33.2	8.9
105	17-Oct-2013	32.86 °N, 117.28°W	McGowan	Present	26.2	12.8
106	17-Oct-2013	32.86 °N, 117.28°W	McGowan	Present	19.8	18.3
107	7-Feb-2014	32.86 °N, 117.28°W	McGowan	Present	29.0	12.8
108	7-Feb-2014	32.86 °N, 117.28°W	McGowan	Present	23.2	13.9
109	20-Mar-2014	32.86 °N, 117.28°W	McGowan	Present	27.1	10.0
110	29-Mar-2014	32.86 °N, 117.28°W	McGowan	Present	29.6	8.9
111	4-May-2014	32.86 °N, 117.28°W	McGowan	Present	29.6	8.9
112	4-May-2014	32.86 °N, 117.28°W	McGowan	Absent	25.3	8.9
113	31-May-2014	32.86 °N, 117.28°W	McGowan	Absent	22.3	12.2
114	31-May-2014	32.86 °N, 117.28°W	McGowan	Absent	25.0	12.2

Table 2.6. ROV Honu surveys

#	Date	ROV Coordinates	Location	Survey Depth Range	<i>D. opalescens</i> Embryo Depth Range
1	1 Aug, 2012	32.80 °N, 117.31 °W	Pacific Beach	38.2-75.1 m	Absent
2	30 Aug, 2012	32.86 °N, 117.28°W, 32.93 °N, 117.30 °W	McGowan Site, Del Mar	33.4-91.5 m 40.6-94.4 m	33.4-91.5 m Absent
3	30 Sep, 2012	32.95 °N, 117.28 °W	Solana Beach	22.0-40.0 m	22.0-40.0 m
4	5 Jan, 2013	32.86 °N, 117.28 °W, 32.80 °N, 117.31 °W	McGowan Site Pacific Beach	28.5-71.0 m 39.9-70.8 m	28.5-33.4 m Absent
5	12 Feb, 2013	32.86 °N, 117.28 °W	McGowan Site	11.2-65.1 m	11.3-55.3 m
6	5 Mar, 2013	32.86 °N, 117.28 °W	McGowan Site	21.0-77.4 m	24.7-64.1 m
7	26 Mar, 2013	32.86 °N, 117.28 °W	McGowan Site	19.0-78.9 m	21.2-68.4 m
8	14 Jun, 2013	32.86 °N, 117.28 °W	McGowan Site	16.1-75.9 m	21.6-40.6 m
9	26 Jun, 2013	32.86 °N, 117.28 °W, 32.80 °N, 117.31 °W	McGowan site Pacific Beach	13.2-74.8 m 19.6-80.3 m	24.9-48.3 m Absent
10	30 Jul, 2013	32.86 °N, 117.28 °W,	McGowan Site Point Loma	11.2-82.8 m 31.2-50.5 m	10.3-65.5 m 31.2-50.5 m

Table 2.6. ROV Honu surveys (Continued).

#	Max [O ₂] (µM)	Max pH	O ₂ /pH Max Zone Depth (m)	Squid Max Density Depth (m)	Squid Max Density (Caps•m ⁻²)	Squid Avg Density (Caps•m ⁻²)	Est. Bed Area (m ²)	Est. Emb•C ap ⁻¹	Est. Total Embryo Count
2	250.3-266.3	8.00-8.06	33.4-43.1	42.0	1.5	0.063	569,720	129	4,630,114
3	250.6-276.9	8.03-8.11	12.5-32.0	28.1	350.1	7.919	516,852	128	523,897,726
4	190.3-194.2	7.88-7.89	28.5-38.2	29.2	5.2	1.548	15,494	141	3,381,844
5	254.8	8.01	11.3-29.7	28.7	3.7	0.158	294,949	153	7,130,097
6	185.3-199.1	7.86-7.89	21.0-34.8	29.2	9.7	0.267	371,430	162	16,065,833
7	160.2-184.0	7.81-7.86	26.6-31.2	27.9	71.4	3.714	731,642	171	464,661,444
8	202.6-212.5	7.89-7.90	19.1-26.1	28.0	7.9	0.183	78,494	219	3,145,804
9	169.7-182.4	7.83-7.88	13.3-19.4	27.8	21.2	1.859	193,653	227	81,720,210
10	204.6-239.6	7.90-7.98	10.3-22.5	24.9	146.7	4.365	317,686	254	352,221,645

Table 2.7. ROV Triton surveys.

#	Date	ROV Coordinates	Location	<i>D. opalescens</i> (embryos)	Depth Range
1	3 Jul, 2012	32.95 °N, 117.37 °W	Slope off McGowan Site	Absent	394-399 m
2	7 Dec, 2012	32.88 °N, 117.36 °W	Slope off McGowan Site	Absent	98-358 m
3	8 Dec, 2012	32.95 °N, 117.32 °W	Slope off of Del Mar	Absent	75-322 m

CHAPTER 3.

Environmental pH and [O₂] influence the Embryogenesis and Statolith Development of Embryonic Squid *Doryteuthis opalescens*

Abstract

Doryteuthis opalescens is an important forage species for the inshore ecosystems of the California Current System. The inshore environment is expected to have lower pH and [O₂] into the future, potentially impacting the development of seafloor-attached encapsulated embryos. In the laboratory setting, *D. opalescens* capsules and encapsulated embryo response to environmental pH and [O₂] was tested. Two experiments were performed. In Experiment 1, embryo capsules were exposed to a treatment of high pH (7.93) and [O₂] (242 μmol kg⁻¹) or a treatment of low pH (7.57) and [O₂] (80 μmol kg⁻¹). Embryos exposed to the low pH and low [O₂] treatment had a 16.7 % longer development time, remained at less developed stages more often and had 54.7% smaller statolith area, independent of embryo size. To tease apart the magnitude of single stressors on squid-embryo development, a second experiment exposing encapsulated embryos to low pH (7.56) only or low [O₂] (85 μmol kg⁻¹) only was conducted. In Experiment 2, embryos (dorsal mantle length) and statoliths were bigger and yolk reserves were smaller in the low pH only treatment relative to those in low [O₂] only treatment. In addition, there were capsular effects (capsular environment affected embryos) throughout both Experiment 1 and 2. These results suggest that *D. opalescens* likely have variable developmental times and fitness within their habitat, driven by different responses to environmental [O₂] than environmental pH (pCO₂). In nature, it is

possible that this contributes to patterns of continuously hatching squid embryos that occur in the Southern California Bight.

Introduction

Market squid, *Doryteuthis opalescens*, ranges from British Columbia, Canada continuously through to Baja California, Mexico (Okutani and McGowan 1969, Jereb et al. 2010). Adults migrate from feeding grounds centered in the mesopelagic and upper slope (Miller et al. 2008) to spawn on the seafloor of the continental shelf (Jereb et al. 2010, Zeidberg et al. 2011a). These migrating adults and their reproductive output are an important ecological link, transferring energy from the slope to the epipelagic waters above and on the seafloor of the continental shelf of the California Current System (CCS). They are also a forage species, providing food for many federally protected species of fish (Morejohn et al. 1978) and tetrapods (Sweeney 2011). *D. opalescens* constitute ~ 61% of the total biomass of all take of all species captured by commercial fishers in California, 28 % of the total ex-vessel value, \$68.3 million (Protasio 2013). In a recent study of squid embryos in central and northern California, USA, most capsules were observed at depths ranging from 20-70 m (Zeidberg et al. 2011a), with the highest levels of abundance in the Southern California Bight (SCB). On the shelf, squid chemically adhere embryo capsules onto a variety of substrates (sandy plains, rocky habitat, kelp, artificial structures, submarine canyon walls) where they develop on or near the seafloor (Navarro et al. 2013, Chapter 2).

Inshore habitats are exposed to the upwelling of low temperature/low oxygen/low pH waters from further offshore (Feely et al. 2008). One-time sampling of embryo beds

during the fishing season from central and southern California, USA purports that squid select a narrow temperature range of 10-14 °C for spawning (Zeidberg et al. 2011a) relative to the range of viable temperatures for which they can develop 8-20 °C (Zeidberg et al. 2011b). Continuous sampling of *D. opalescens* embryo beds at La Jolla, USA show that the temperature ranges from 10-16 °C at shallow parts of the embryo bed (30 m) and rapidly decreases with depth on the shelf (Navarro et al. 2013). Temperatures of waters between 7-17 m depth can vary at semidiurnal, diurnal, and episodic timescales with the strongest variation experienced at the semidiurnal and diurnal scales (Frieder et al. 2012). Temperature usually fluctuates with pH, salinity, and [O₂] below the thermocline in the Southern California Bight (Alin et al. 2012).

Squid-embryo habitat is exposed to pH units ranging from 7.65 to 8.1 and [O₂] levels from 70-240 μmol kg⁻¹ (Navarro, Chapter 2). Since pre-industrial revolution times, the surface waters of the California Current System have declined by 0.1 pH units (Hauri et al. 2009, Takeshita et al. 2014) and the oxygen minimum zone upper boundary has shoaled by as much as 90 m over the last 25 years (Bograd et al. 2008, 2014, McClatchie et al. 2010, Booth et al. 2012, 2014). Globally [O₂] is expected to decline by 1% to 7% in surface waters over the next century (Keeling et al. 2010). At *D. opalescens* embryo habitat, pH is expected to decrease by 0.11-0.13 units by 2060 (Takeshita et al. 2014). It is not known how *D. opalescens* embryos develop with exposure at the lower, and presumably, more stressful end of the range for environmental pH and [O₂].

The *D. opalescens* embryo stage is susceptible to environmental change because encapsulated embryos 1) are attached to the seafloor and unable to escape exposure to intruding low oxygen and low pH waters and 2) each embryo has a fixed energy reserve

(i.e. yolk) for embryogenesis and regulation of internal pH. Loliginid embryos are dependent on aerobic metabolism and can be negatively affected by low $[O_2]$ (Roberts 2005, Zeidberg et al. 2011a). Energy reserves are required to excrete costly wastes induced by exposure to low-pH environments. In contrast to all other life stages (i.e. paralarva, juvenile, adult) *D. opalescens* embryos cannot swim to better-oxygenated, high-pH waters (e.g. surface). Other life stages can vertically migrate daily, potentially limiting their exposure to harsh environments to hours instead of days. In addition, at these later life stages, *D. opalescens* can expand their energy budgets by consuming more food (i.e. energy source is not fixed). Juvenile and adult life stages can consume 35-80% of their body weight each day to meet their metabolic needs (Chen et al. 1996). *D. opalescens* can spawn at all times of the year in the Southern California Bight (Navarro et al. 2013). Chronic (month-long) exposure of encapsulated embryos to low environmental levels of pH and $[O_2]$ were chosen to best match the time scale of development.

Two experiments were conducted to test for effects of chronic exposure to realistic low environmental pH and $[O_2]$ conditions on embryo development duration, metabolism, and sensory organ structures (statoliths). Developmental duration of embryogenesis is measured as the time from being laid to just prior to hatch (i.e. stage 9 to 28; Fields 1965). Metabolic effects are inferred by evaluating embryo yolk utilization and sensory organ impacts were inferred by morphometric shape analyses of statoliths. Embryo growth rate was hypothesized to be slowed and developmental duration to be lengthened under low environmental $[O_2]$ and pH (pCO_2) conditions. The $[O_2]$ treatment was expected to induce metabolic suppression and slow the rate of growth. However, the overall size at the near-hatch stage was not expected to differ from that in embryos

exposed to high [O₂] levels. Cephalopod embryo masses have been shown to be highly impacted by environmental [O₂] levels (Cronin and Seymour 2000, Roberts et al. 2005, Gutowska and Melzner 2009). Further, lower environmental [O₂] levels are sufficient to induce a coping response (e.g. metabolic depression) in most cephalopod embryos including squid embryos (Robin et al. 2014).

The pH treatment is expected to affect embryo and statolith size (Kaplan et al. 2013) and composition (Lacoue-Labarthe et al. 2011, Navarro et al. 2014). Embryo statolith development was hypothesized to be affected by pH (high *p*CO₂) treatments but not [O₂] treatments. *D. opalescens* statoliths are made of aragonite (Warner et al. 2009) and are vital for sensing gravity, balance, and movement (Arkhipkin and Bizikov 2000, Arkhipkin 2005, Kaplan et al.). These senses are critical throughout all life stages of the squid, especially just after hatching during the critical stage (Chen 1996), but it is unknown whether statolith development in *D. opalescens* is affected by environmental pH and [O₂] or is decoupled from the environment.

Statoliths are enclosed within several biological compartments including the statocysts, embryo, chorion, and capsule that separate them from the seawater environment and can decouple their development from environmental conditions (Navarro et al. 2014). Low environmental pH/high *p*CO₂ is known to affect statolith composition (Lacoue-Labarthe et al. 2011, Navarro et al. 2014) and size for other species of lolignids (Kaplan et al. 2013). Based on these studies and studies on the otoliths of white seabass (Checkley et al. 2009) and cod (Maneja et al. 2013), statoliths of the squid were hypothesized to grow larger in the low environmental pH treatments than in the low [O₂] treatment.

Research Design and Methods

To test the hypotheses, newly collected *D. opalescens* embryo capsules were placed into two separate laboratory experiments using the Multiple Stressor Experimental Aquarium at Scripps (MSEAS; Bockmon et al. 2013). Each experiment contained two treatments; each treatment consisted of two replicate tanks. Fixed conditions across all experiments were: temperature = 11.3 °C, light = 3000 lux (12 hr:12 hr light:dark daily cycle), salinity = 33.4 PSU. Experiment 1 compared high and low [O₂] and pH levels, as they vary together with a positive correlation in the field (Nam et al. 2011, Send and Nam 2012, Frieder et al. 2012). This experiment used embryo capsules collected from the field at La Jolla, USA (cohort 1; n=24; 32.86 °N, 117.27 °W, 30 m depth) and laid in captivity at Scripps (cohort 2; n=16) by squid captured off Del Mar, USA (32.96 °N, 117.28 °W). The first treatment, termed “high pHOx” had an [O₂] level at 242.0 μmol/kg (± 12.7) and a pH of 7.93 (± 0.058). In the second treatment, “low pHOx,” [O₂] level was 80.4 μmol/kg (± 18.7) and pH level was 7.57 (± 0.066). Experiment 1 treatment values are characteristic of upwelling (low pHOx) and relaxation (high pHOx) events in the region (Send and Nam 2012). Experiment 2 used embryo capsules (n = 80) collected from La Jolla, USA (32.87 °N, 117.25 °W). This experiment tested [O₂] and pH each as a single stressor (i.e. low [O₂] only or low pH only). The “low pH” only treatment pH level was 7.56 (± 0.028) and [O₂] level was 241.4 μmol/kg (± 8.4) whereas the “low [O₂]” only treatment [O₂] level was 84.7 μmol/kg (± 10.6) and pH level was 7.92 (± 0.054). In both experiments, pH was controlled by manipulating pCO₂ and alkalinity remained relatively constant (total alkalinity range = 2214-2244 μmol/kg; Navarro et al. 2014).

In nature, squid embryo development duration is temperature dependent. When *D. opalescens* embryos are exposed to environmental temperatures of 16 °C, development lasts ~21 d, in contrast to 30-35 d duration when exposed to 13.6 °C (Jereb et al. 2010). In the lab, these same temperatures result in a much longer development duration (Zeidberg et al. 2011b), suggesting that other factors including abiotic (e.g. surge, light levels) and biotic (e.g. predator and prey abundance) conditions also influence embryo development time. The first finding of our study impacted our experimental design in that the low pH_{OX} treatment duration lasted 5 d longer than the high pH_{OX}. Therefore, half of the low pH_{OX} treatment capsules were collected at the same time as all of the high pH_{OX} treatment capsules and half of the low pH_{OX} treatment were collected 5 d after the initial collection near hatching (n=8 capsules per treatment). In addition, the most developed low pH_{OX} and high pH_{OX} embryos were used to compare size and statolith variation among treatments for embryos at the same developmental stage (n=10 capsules per treatment). Experiment 2 treatments (low pH only and low [O₂] only) had the same developmental duration. Half of the capsules were removed after 27 d (n=20) and the remaining capsules were removed at the end of development after 32 d (n=20). Upon removal, embryo capsules were immediately photographed, and then dissected into subsamples for morphological analyses. A single capsule is packed with hundreds of embryo-filled chorions (usually one embryo per chorion), and each embryo has two statocysts, each containing a single statolith. Per capsule, approximately 50 embryos and their chorions from the middle position of the capsule (Steer et al. 2002) were preserved in 5% formalin in filtered seawater (50 µm). Approximately 50 embryos were frozen at -80 °C for statolith analyses. The total number of embryos per capsules was quantified.

Capsule, chorion, embryo, and statolith data were collected using photo microscopy (Canon PC1305, 4416 x 3312 pixels). Adobe Photoshop CS5 was utilized to adjust light levels and enhance sharpness of edges. Capsule length and width dimensions and chorion maximum and minimum diameters were measured. Embryos were extracted from the chorion and photographed while flat with the dorsal side up. The dorsal side was identified based on the position of fins and, if mature enough, chromatophore patterns. Embryo parameters measured include 1) total length (TL), 2) head width (HW), 3) dorsal mantle length (DML), 4) yolk length (YL) and 5) yolk diameter (YD; Figure 3.1.). Yolk length, height (h), diameter and radius (r) were used to estimate yolk volume (YV).

$$YV = (\pi r^2 (h/3)) + 0.5 ((4/3) \pi r^3)$$

$$r = (0.5YD)$$

$$h = YL - r$$

Yolk volume was converted to wet-weight mass (O'Dor et al. 1986) and then to calories (Giese 1969) as was done in Vidal et al. (2002).

$$\text{Wet-weight mass} = 1.036 \text{ mg/ mm}^3 \text{ (O'Dor et al. 1986)}$$

$$\text{Calories} = 1.71 \text{ cal/ mg (Giese 1969)}$$

To assess differences among treatments, *development was categorized* (*sensu lato* Portner et al. 2010) based on developmental stages, and treatments were compared at the same developmental category. Squid lack numerous discrete developmental signposts, have continuous progression in development (Portner et al. 2010), and have

plasticity in terms of time of development (Zeidberg et al. 2011b). To standardize comparisons, allometric measurements (Zeidberg 2004, Katsanevakis et al. 2006, Shea and Vecchione 2010) were used to categorize embryo development and were further resolved using embryonic stages (Arnold 1965, Segawa et al. 1988, Fields 1965). DML:HW ratio was an allometric means to determine developmental category (Table 3.1., Figure 3.2.). Developmental category was further resolved using embryonic stages described by Arnold (1965; *D. pealii*) and Segawa et al. (1988; *L. forbesi*). Fields (1965; *D. opalescens*) was used to a lesser extent because his work was based on changes associated with developmental duration rather than key stages in organogenesis.

Statoliths were dissected from the late developmental stage embryos (\geq allometric category 5) and removed from statocysts. To remove remaining tissue on the statoliths, tissue was dissolved using a dilute bleach solution and then the statoliths were rinsed three times with distilled water (Kaplan et al. 2013). Statoliths were photographed via photo microscopy and measured by length, width, and area. All measurements were taken using Image J Version 1.47.

For analyses of samples (i.e. capsules, chorions, embryos) under the same exposure duration to treatments, tanks were considered replicates for statistical testing of the hypotheses. All data were first examined for variance homogeneity and tested for normality by means of residual analysis. Experiments 1 and 2 were tested separately. For experiment 1, since two cohorts were used, treatment effects were nested within cohort using a one-way hierarchical ANOVA (Bettington and Thanye 1994). Further, tank and exposure effects were nested within each treatment and cohort and capsule effects were

nested within each cohort, treatment, and exposure group. Capsule effects were considered “random” and treatment, cohort, tank, and exposure effects were considered “fixed.” In Experiment 2, a one-way hierarchal ANOVA was used to test for effects of treatment, exposure duration, tank, and capsule on embryonic structures. Tank and exposure duration effects were nested within each treatment and capsule effects were nested within each treatment and exposure group. Capsule effects were considered “random” and treatment, tank, and exposure effects were considered “fixed.”

For analysis of embryos and statoliths at the same developmental stage, Experiments 1 and 2 were tested separately. Embryos from the same capsule were considered replicates. Embryo data were tested at each developmental category. For Experiment 1, one-way hierarchal ANOVA was used to test treatment effects. Cohort, exposure duration, and capsule effects were nested within each treatment. Treatment, exposure duration and cohort were tested as “fixed” effects and capsule was tested as a “random” effect. Experiment 2 was tested in a similar way with the exception that the cohort effect was not tested (all embryos in Experiment 2 were from the same cohort). Effects of treatment on statolith structure were tested by one-way hierarchal ANOVA. Statoliths tested from Experiment 1 were all from cohort 1. The treatment effect was tested as “fixed” and capsule effect as “random.” Capsule effect was nested within the treatment effect. Statistical analyses were conducted using JMP (Version 11 Pro) software.

Results

Cohorts

The number of embryos per capsule in Experiment 1 did not differ between cohorts (i.e. cohort taken from the field compared to the cohort laid within the lab). As such, these cohorts are thought of as coming from a similar population of squid. In contrast, the squid populations between experiments are likely different to some degree because the number of embryos per capsule of the pooled cohorts from experiment 1 (165 ± 7.3) compared to those from experiment 2 (190 ± 4.9) were significantly fewer by 15% ($F_{1,112} = 8.585$, $P = 0.004$). This may reflect a different capsular environment between experiments as more embryos per capsule may mean each embryo is exposed to higher levels of $p\text{CO}_2$ and lower levels of pH and $[\text{O}_2]$. Experiment 1 capsular structures differed between cohorts. The cohort collected from the wild had significantly more capsule swelling (associated with development time) than the aquarium cohort. These capsules were longer (39.7%; $F_{3,22} = 10.043$, $P = 0.0008$) with more volume (11.4%; $F_{3,22} = 6.265$, $P = 0.0070$) than those from the aquarium. Although these chorions were not statistically different in volume between cohorts the chorions looked bigger from the wild cohort suggesting that the chorion data set may have lacked necessary statistical power.

Experiment 1

There were no treatment effects on capsule dimension or chorion diameter. However, treatment and cohort effects were evident for embryonic structures (Figure 3.3.). The embryos from the high pH_{OX} treatment were longer, evidenced by the DML being 44.2% longer ($F_{1,23} = 118.304$, $P < 0.0001$), and had a HW that was 16.1% narrower ($F_{1,23} = 31.293$, $P < 0.0001$; Figure 3.3.). Further, the external yolk sac was much smaller in the high pH_{OX} treatment. The yolk volume was 59.2% reduced in the high pH_{OX} treatment relative to the low pH_{OX} treatment ($F_{1,23} = 137.113$, $P < 0.0001$). Embryos in the high pH_{OX} treatment had a similar TL as those from the low pH_{OX} treatment ($F_{1,23} = 0.012$, $P = 0.91$). Beyond, treatment effects there were several nested effects. The two cohorts had distinct DMLs and cohort 1 embryos had DMLs that were 31.4% longer ($F_{2,23} = 31.599$, $P < 0.0001$) and similar HW, YV, and TL in comparison to cohort 2. There were no tank effects. For both cohorts, embryos that were allowed more exposure time to the low pH_{OX} treatment had longer DMLs ($F_{8,23} = 3.770$, $P = 0.0202$), narrower HWs ($F_{8,23} = 4.168$, $P = 0.0114$), smaller YVs ($F_{8,23} = 3.789$, $P = 0.0174$) and similar TLs. Capsule effects significantly impacted embryo DML ($F_{16,23} = 2.314$, $P = 0.0070$), HW ($F_{16,23} = 1.825$, $P = 0.0414$), and YV ($F_{16,23} = 3.080$, $P = 0.0006$) but not TL.

Experiment 2: How do low pH and low [O₂] effects compare to each other?

To better understand the effects of Experiment 1, Experiment 2 tested whether or not pH and [O₂] had distinct effects on embryo development. In Experiment 2, the capsules and chorions were not structurally distinct between treatments. Treatments

distinctly affected embryo DML, TL, HW, and YV (Figure 3.4.). The embryos had 16.3% longer dorsal mantle lengths in the low pH treatment ($F_{1,79} = 136.722$, $P < 0.0001$) with HWs that were 5.3% wider ($F_{1,79} = 8.259$, $P = 0.0053$) compared to the low [O₂] treatment. In the low pH treatment YV was reduced by 126.5% reduced ($F_{1,79} = 268.624$, $P < 0.0001$) and TL was 4.5% shorter ($F_{1,79} = 34.281$, $P < 0.0001$). Older embryos (32-d exposure) had longer DML ($F_{4,79} = 288.759$, $P < 0.0001$), reduced YV ($F_{4,79} = 91.558$, $P < 0.0001$) and longer TL ($F_{4,79} = 16.784$, $P < 0.0001$). Exposure duration did not affect the HW between groups. For low pH treatment, the YV of the 32-d exposure group (0.147 mm³) approached zero and was reduced by 506.7% compared to the 28-d exposure group (0.842 mm³). For the low [O₂] treatment 32-d exposure group, there was a 79% reduction of the YV (0.842 mm³) compared to the 28-d exposure group (1.515 mm³).

There was a tank effect for DML ($F_{2,79} = 10.042$, $P < 0.0001$), and YV ($F_{2,79} = 3.856$, $P = 0.0067$), largely driven by differences between tanks from the pH treatment only at 28 days of exposure (Appendix, Figure 3.A1.). In contrast, at 32 days, there was no difference in embryo traits between pH treatment tanks so we suspect that the tank effect found at 28 days of exposure may not be biologically significant. Further, the environmental conditions between the pH treatment tanks were statistically similar (Navarro et al. 2014).

Capsules effects were observed for DML ($F_{54,79} = 4.684$, $P < 0.0001$), HW ($F_{54,79} = 10.005$, $P < 0.0001$), YV ($F_{54,79} = 6.011$, $P < 0.0001$), and TL ($F_{54,79} = 7.102$,

$P < 0.0001$). These differences are biologically significant and are likely caused by the capsular and chorion membranes and maternal effects (see Navarro et al. 2014).

Developmental Duration and Treatment Effects by Developmental Category

All developmental categories were represented for Experiment 1 (Figure 3.5.) and 2 (Figure 3.6.). However, development was highly variable within each treatment and each treatment had a high range of categories at any given exposure treatment (Figure 3.5. and 6). Thus, the duration of embryogenesis is specific to each embryo. The total development time was compared for the embryos between treatments at the time when each developed to category 6. The absolute development time for Cohort 2 was 35 days in the low pHOx treatment. Cohort 2 embryos exposed to the high pHOx treatment were estimated to have taken 30 days to develop. Cohort 1 embryos at category 6 in the high pHOx treatment developed 5 days prior to those from the low pHOx treatment. Embryos chronically exposed to low pHOx develop at a rate of 16.7% slower than those chronically exposed to high pHOx. The embryos developed to category 6 at relatively the same time for both the low pH only and low [O₂] only treatments. However, more embryos were developed to category 6 in the low pH treatment (Figure 3.6.). Embryos were approximately 4 days old prior to being placed into the experiment (development stage 9, Arnold 1965). Both these treatments induced slower development than the high pHOx treatment. Embryos in these treatments were estimated to reach category 6 about 32 d after being laid. Embryos were sampled prior to hatching (at 32 d) in order to ascertain embryo position within the capsule. Exposure periods beyond 32 d would have

resulted in hatching and thus information about the position of individual embryos would have been lost.

Embryo Size by Developmental Category

Treatments did not significantly affect category-6 embryos in Experiment 1 (Figure 3.7.). However, there was a trend for the DML and HW to be longer in the high pHOx treatment. Among the five categories tested (category 2-6), embryo DML from the high pHOx treatment was significantly longer only at category 3, embryo YV was significantly larger at category 3, 4 and 5 and embryo TL was distinctly longer at category 4 (Figure 3.7., Table 3.A1.A.). Embryo HW was similar among treatments for all categories.

The cohorts were similar in size metrics with a few exceptions. Cohort 1 had a longer DML at category 5. Cohort 2 had a longer DML at category 4 and a longer TL and smaller YV at category 3 (Figure 3.7., Table 3.A2.). These variations suggest that each cohort differs slightly in the timing of their metabolic processes. Based on examination of exposure duration in all possible categories (1-4, low pHOx only) exposure duration did affect YV at category 3 and TL at category 2 (Table 3.A3.A). Capsule effects were significant for DML at category 5, YV at category 3, 4 and 6, HW at category 4 and TL at category 3, 4, 5 (Table 3.A4.A).

Treatments significantly affected embryos at category 6 in Experiment 2 (Figure 3.8.). Embryos from the low pH only treatment had a longer DML, a wider HW and a

smaller yolk sac (Figure 3.8., Table 3.A1.B). Embryo TL was similar between treatments. Among the other four categories tested (category 2-5), embryo DML from the low pH only treatment was significantly longer at category 2, 4 and 5, embryo YV was smaller at category 2, 3, 4 and, embryo HW was distinctly wider at category 2, 3, 4 and 5, and embryo TL was smaller at category 5 only relative to the low [O₂] only treatment (Table 3.A1.B). Examining all possible categories (3-6), the 32-d exposure treatment embryos had longer DML at categories 4, 5 and 6, smaller YV at category 5 and 6, wider HW at categories 5 and 6, shorter TL at category stage 4 and longer TL at category 6 relative to the 28-d exposure treatment (Figure 3.8., Table 3.A3.B.). Strong capsule effects were present across all developmental categories (2-6) affecting DML, YV, HW and TL (Table 3.A4.B).

Yolk Utilization

For Experiment 1 and 2, treatments presumably impacted the embryo's ability to access their yolk (caloric reserves; Figure 3.9.). Yolk utilization is vital for development and embryos that fail to utilize their yolk will die. Further, embryos that utilized these caloric reserves had larger DMLs indicating that they may have been able to use these calories, at least in part, towards growth. In Experiment 1, embryos from the low pH_{OX} treatment had larger YVs at categories 3-5 and shorter DMLs at developmental category 3 (Figure 3.7., 3.9A.). In Experiment 2, embryos from the low [O₂] treatment had shorter DMLs at categories 4-6 and larger YVs at categories 2-6 (Figure 3.8., 3.9B.). For both experiments, utilization of caloric reserves is directly and positively correlated to DML (size of the squid embryo; Table 3.2.).

Statoliths

In Experiment 1, statoliths from category-6 embryos in the low pH_{OX} treatment were 54.7% smaller in area ($F_{1,15} = 18.469$, $P = 0.0014$), 40.3% shorter in length ($F_{1,15} = 24.726$, $P = 0.0005$), and 7.3% narrower in width ($F_{1,15} = 8.014$, $P = 0.0171$) compared to those from the high pH_{OX} treatment (Figure 3.10A.). Since the DML of the most developed embryos (at category 6) was similar between treatments, the statolith size difference between these embryos is not attributable to overall embryo size difference. Strong capsule effects were present for each statolith morphometric (area, $F_{12,15} = 4.228$, $P = 0.0002$; length, $F_{12,15} = 3.773$, $P = 0.0005$; width, $F_{12,15} = 4.593$, $P < 0.0001$).

In Experiment 2, pH and [O₂] single stressors did not affect statoliths similarly. Statoliths from embryos in the low [O₂] treatment were 31.9% smaller in area ($F_{1,19} = 37.722$, $P < 0.0001$), 17.6% shorter in length ($F_{1,19} = 43.846$, $P < 0.0001$), and 12.7% narrower in width ($F_{1,19} = 29.645$, $P < 0.0001$) compared to those from the low pH treatment (Figure 3.10B.). These effects might be attributed to overall embryo size because embryos were also bigger in the low pH treatment than in the low [O₂] treatment. Also, capsule effects were found for each statolith morphometric trait (area, $F_{16,19} = 4.698$, $P < 0.0001$; length, $F_{16,19} = 3.623$, $P < 0.0001$; width, $F_{16,19} = 4.216$, $P < 0.0001$).

Results Summary

This study shows for the first time, that chronic exposure (≥ 24 d) to low pH, low [O₂], and low pH_{OX} effect squid embryo development duration, growth, yolk utilization, and statolith development. Synchronous exposure to 7.55 pH ($p\text{CO}_2 = 1440 \mu\text{atm}$) and 90

$\mu\text{mol/kg O}_2$ slowed embryogenesis duration by 16.7%. Many embryos were remained at early development categories. Embryos from the high pHOx treatment utilized, 59.2% of their yolk, were 44.2% longer in DML and had 54.7% larger statoliths (area). By comparing embryos from each treatment at developmental category 6 (near-hatch), we found that statoliths from the low pHOx treatment were distinctly smaller (across all morphometric traits; area by 54.7%, length by 40.3%, and width by 7.3%) and indicate that the treatment affected statolith development (not developmental delay). In contrast, DML, YV, HW, and TL were similar between treatments and thus we infer that these morphometric differences were likely due to developmental delays (not within-stage developmental differences).

In addition, squid embryos are affected by pH and $[\text{O}_2]$ as single stressors. Embryos exposed to low pH had 16.3% longer DMLs, 5.3 % wider HWs, 4.5% longer TLs, and 126.5% smaller YV than those exposed to low $[\text{O}_2]$. Comparing embryos from the low pH treatment at the same developmental category 6 to those from the low $[\text{O}_2]$, the DML was longer, HW was wider, and they had smaller yolk sacs (Figure 3.8., Figure 3.9.B) but the TL was similar. Statoliths from embryos in the low $[\text{O}_2]$ treatment were 31.9% smaller, 17.6% shorter, and 12.7% narrower compared to the low pH treatment. The duration of embryogenesis was similar between treatments. Distinct embryo DML, HW, and YV and statolith area, length and width between treatments reflect developmental differences (not development delays).

Discussion

Environmental O₂ effects of on *D. opalescens* embryos have not been reported previously. Embryos were hypothesized to be more negatively affected by low [O₂] conditions compared to those exposed to low pH (high pCO₂) conditions. Strathmann and Chaffee (1984) provided strong evidence that molluscan-gelatinous embryo masses are highly impacted by stressful environmental [O₂] levels; however, no evidence of an effect on the outer embryonic structures (capsules length and chorion diameter) at the [O₂] levels tested was found. While the levels of pH, pCO₂, and [O₂] within these structures is not known, evidence was found that embryos exposed to low environmental [O₂] are smaller in size both in terms of the DML and statolith area, length, and width compared to embryos exposed to low environmental pH. The positive relationship between embryo and statolith size has been found in other species of loliginids (Steer et al. 2003, Villanueva et al. 2007). Further, smaller size is a common response to low-environmental [O₂] in marine invertebrates (Cohen and Strathmann 1996, Strathmann and Strathmann 1995, Strathmann and Chaffee 1984) and may be indicative of metabolic depression, as has been found for cephalopods when exposed to high temperatures and low pH (Rosa et al. 2012, 2013, 2014)

The null hypotheses for embryo exposure to low environmental pH only (high pCO₂) was that embryo size and development duration would be relatively unaffected compared to those exposed to low [O₂]. Although effects of environmental pH and pCO₂ can be additive (Hu et al. 2012, 2013, 2014, Rosa et al. 2012, 2013, 2014) the change can be small compared to what already occurs during development within the perivitelline fluid, even when the environment pH is at its highest (Melzner et al. 2009). No evidence

was found to suggest that embryos experienced metabolic depression in the low-pH treatment. Further, embryos from the low pH treatment had the ability to access their caloric reserves at each developmental category (Figure 3.9.B) and appeared able to use yolk for growth as the embryo and statolith size was large compared to the low [O₂] only treatment. Cephalopods such as cuttlefish (Rosa et al. 2013) and squid (Rosa et al. 2012, 2014) had the highest hypoxia threshold and were tolerant to lower pH levels at temperatures near the temperature used in our study. At temperatures greater than our study, Rosa et al. (2012, 2013, 2014) found that reduced pH decreases hypoxia thresholds ultimately leading to metabolic depression. Similarly, Kaplan et al. (2013) gave evidence that, at an environmental temperature of 20 °C and *p*CO₂ level of 2200 μatm, *Doryteuthis pealeii* embryos had reduced size (by ~1%) relative to those exposed to a *p*CO₂ level of 390 μatm.

Further, it is inferred that at the levels tested, no hypoxia threshold was crossed or extracellular acidosis induced. Cuttlefish (Hu et al. 2011a) and squid (Hu et al. 2011b, Hu et al. 2013) have significantly up regulated acid-base regulation when exposed to high *p*CO₂ but no signs that embryos were negatively affected by environmental pH was detected in this study. In addition, the pH/*p*CO₂ treatment did not appear to affect the fluid within the statocyst. The statoliths did not have observable malformations and, other than their large size, appeared normal. Furthermore, their elemental composition was not elevated in uranium:calcium (Navarro et al. 2014), as found with other molluscs if subject to low environmental pH (Frieder et al. 2014). Research conducted on fish saccules (fish analog to statocyst) provides evidence that in fish this sensory organ is highly regulated in terms of pH (Shiao et al. 2005). Statocyst might be expected to have

similar physiological processes as the fish saccules, as the statocyst performs a similar sensory function.

The larger embryo and statolith size and smaller yolk reserves may indicate that embryos exposed to low environmental pH (high $p\text{CO}_2$) develop faster and longer than those from the other treatments, rather than slower. However, this might not be a benefit. Marthy et al. (1976) identified the PVF as an important “tranquilizer,” allowing the necessary time for the embryo to grow. If squid embryo metabolic rate is determined by $p\text{CO}_2$ levels in the perivitelline fluid (PVF), diffusion of high environmental $p\text{CO}_2$ into the PVF may induce premature development causing embryos to exhaust their caloric reserves before hatching. Increased $p\text{CO}_2$ and/or $[\text{HCO}_3^-]$ is hypothesized to cue development in squid embryos (Marthy et al. 1976). If so, then early maturation may result in depletion of the yolk prior to first feeding which leads to increased mortality rates of paralarvae (Vidal et al. 2002).

Interestingly, when the embryos were exposed to chronic low pHOx , embryos slowed development and most did not develop to near-hatch embryos. These later embryos likely experienced metabolic depression. Embryos from the low pHOx had malformations not observed in other treatments that were similar to those found in studies of environmental hypercapnia (Kaplan et al. 2013). However, some did make it to the near-hatch stage, and these embryos had similar morphology (DML, HW, YV, TL) to embryos from the high pHOx treatment (with the exception of having smaller statoliths). Their statoliths had enriched uranium:calcium (Navarro et al. 2014), suggesting that the pH within the statocyst was lower than in the other treatments. This is interesting because the pH likely is highly conserved to preserve the shape of the biogenic carbonate

and its distance to the sensory cells, as is true for the gravitational sensors of fish (Shiao et al. 2005). These findings suggest that these embryos can only develop through chronic exposure to low pHOx.

Hatch Timing

In nature, a spread of hatching times within a cohort may help ensure that at least some of the progeny find adequate sources of food. The high pHOx and low pHOx treatment levels represent a range of levels close to those that are found in nature at 10-90 m depth (Navarro et al. 2013) within embryo beds of southern California. Levels at or close to the high pHOx values tend to be those occurring at shallower depths (upslope) or in Fall/Winter while those levels close to the low pHOx tend to occur downslope at the mid portions of the continental shelf or in Spring and early Summer. Embryo beds can cover vast portions of the seafloor (square kilometers) on the continental shelf at depths ranging from surface waters to 90 m (Zeidberg et al. 2011a, Navarro et al. 2013). It is common for different areas of the bed to experience differing levels of pH and [O₂] (as well as temperature, surge, light, visibility, etc., M. Navarro unpublished). In nature, *D. opalescens* embryos laid from the same cohort over variable depths are predicted to hatch in a continuum starting in shallow waters and progressing to deeper waters over as much as a week in part because of their exposure to differing levels of pH and [O₂]. pH and [O₂] effects are similar to temperature effects, at least in some ways, in that they also slow development duration (Ziedberg et al. 2011b). Squid embryo-developmental duration is highly sensitive to environmental variation.

Although the low [O₂] only treatment was expected to increase embryo development time, embryos were found to have similar development time in both the low [O₂] only and low pH only treatments. Hypercapnia usually affects metazoans by slowing metabolic rates (Gutowska et al. 2008). Metazoans with low metabolic rates often experience metabolic depression, whereas those that have evolved higher metabolic rates (e.g. fish and cephalopods) have done so in part by their tolerance to hypercapnia (Gutowska et al. 2008). In her studies, Gutowska et al. (2008, 2010) found that the body mass of cuttlefish did not vary among pH/pCO₂ treatments. Loliginids (such as *D. opalescens*) have high metabolic rates (O'Dor et al. 1986, O'Dor and Wells 1987). The results of this study showed 16.7% slower development for *D. opalescens* embryos exposed to low pH/O₂. These results are comparable to development delays reported for the embryos of *D. pealeaii* by ~ 7% (Kaplan et al. 2013) and *Loligo vulgaris* by 37% (Rosa et al. 2014) subjected to low pH/ high pCO₂ exposure.

Sensory Development

Impacts to sensory organs can greatly alter the survival rate of larvae (Hanlon et al. 1989). Statocysts function as the gravity-sensing organ of the squid to detect acceleration in the x, y, and z axes (Arkhipkin and Bizokov 2000). In this study statolith size (area, length, width) varied greatly across treatments, as was found in low environmental pH/high pCO₂ (2200 µatm) treatments by Kaplan et al. (2013). However, under the low pH only treatment, the statolith area was larger (not smaller as reported by Kaplan et al. 2013) compared to embryos exposed to low [O₂]. The statolith size difference in Experiment 2 is likely a reflection of the bigger embryo size and may not

affect statocyst function. Previous findings show that environmental pH also affects the size of some fish otoliths (Checkley et al. 2009, Maneja 2013).

In contrast, embryos exposed to low pHOx may not be capable of internal pH regulation, which may lead to smaller statoliths. Low [O₂] in combination with low pH may suppress the metabolism of the embryo so greatly that the embryo cannot transport H⁺ ions across membranes such as the statocyst membrane. Alternatively, metabolic depression can also suppress or delay the release of [HCO₃⁻], and when low [O₂] is combined with low pH (high pCO₂), the effect is enhanced. Statolith size changes, both positive and negative, can have dramatic effects on the fitness of the squid. Although behavior of paralarvae was not tested the statolith results suggest changes in paralarvae behavior could be significant. Specifically, smaller area and length can reduce squid ability to sense jet/sink movements (Arkhipkin and Bozikov 2000). Squid with impaired hunting ability have higher mortality rates at the paralarvae stage, especially through the critical period (Vidal et al. 2002).

Fitness

Surviving the critical period to first feeding is a balancing act between growing large enough to enhance hunting ability (Steer et al. 2003) and retaining enough calorie reserves (yolk) to “buy” the time necessary to learn to hunt (Vidal et al. 2002).

Paralarvae have an internal yolk sac with small amounts of reserves (relative to outer yolk sac). Although the embryos from the low pH treatment were large, interpretation that they had the highest fitness may not be correct, because these embryos also nearly exhausted their outer yolk sac prior to hatch. Hatching is likely an energy-intensive

process. In contrast, embryos from the high pH_{OX} treatment had much more yolk remaining at the near-hatch category (Table 3.2.). Embryos from the low pH treatment would have much less time to “learn” than embryos from other treatments and, without an adequate caloric reserve, we predict that embryos exposed to low pH_{OX} have a higher mortality rate compared to those exposed to high pH_{OX}. On the other hand, embryos can also have a lower fitness if they do not use their yolk. Embryos that are smaller with full internal yolk reserves can be at a disadvantage if they are too small (Vidal et al. 2002). Further studies are needed to resolve what size and yolk reserve combination indicates best fitness.

Presumably, overall fitness for embryos in the low pH_{OX} treatment was lower than those from the high pH_{OX} treatment, as many embryos remained at early developmental categories (Figure 3.5.). Although they were not dead, many embryos were at the same development stage as found for younger embryos. Most did not make it to the near-hatch category and we predict that in nature, embryos exposed to low pH_{OX} conditions would have a much higher mortality rate. Also, those that did make it to the near-hatch category did so with smaller statoliths than those from the high pH_{OX} treatment. Although still untested, smaller statoliths may impair statocyst function, which would lower their fitness by impairing their ability to hunt as paralarvae.

The variability in phenotypic and developmental timing responses to low pH_{OX} conditions observed in this experiment may reflect a high capacity for plasticity and adaptation. However, further field sampling is needed to understand whether climate change is selecting for embryos resistant to low pH_{OX} conditions. Experimental evolution in short-generation phytoplankton suggests that while initial physiological

responses to low pH may be negative, mutation-driven evolution from single genotypes can occur within 500 generations and can include changes in regulation of genes involved in growth and pH regulation (Lohbeck et al 2014). In marine populations with large population sizes and high dispersal capacities, natural selection on standing genetic variation may bring about population-level adaptation to low pH conditions much more rapidly (Pespeni et al 2013). *D. opalescens* is characterized by a large, highly connected population (Reichow and Smith 2001), with high dispersal capacity as adults and a short generation time (100 generations of *D. opalescens* can occur in 50 years, assuming average life span of ~6 months; Yang 1986, Jackson 2002). These life history characteristics may allow adaptation in squid to occur quickly relative to other metazoans. Indeed, to persist *D. opalescens* will have to adapt to changes in their habitat occurring due to the expanding OMZs (Bograd et al. 2014, McClatchie et al. 2010, Booth et al. 2014), intensified upwelling, and ocean acidification (Gruber et al. 2012). It is not known how these environmental impacts affect the role of *D. opalescens* in the ecosystem nor is it known how these effects impact the commercial fishery. Environmental assessment should first account for embryo habitat changes on the shelf, with particular attention to the depth distribution of embryo beds because pH, [O₂], temperature, salinity, and current speed change with vertical distances is two orders of greater in magnitude relative to horizontal distances. Second, the assessment should monitor changes along the entire species range as squid are considered one population (Baja to British Columbia; Jereb et al. 2010).

Squid Response to Natural Environmental Stressors in southern California

In southern California, *D. opalescens* are found on the shelf year round (Navarro et al. 2013). Embryos in the field are usually found at between 10 and 14.5 °C with peak densities at 11.5 °C in southern California (Zeidberg et al. 2011a). It is unknown whether or not squid are actively selecting sites based on temperature, based on another environmental factor associated with temperature, or based on an environmental factor independent of chemistry. However, if they do select their spawning sites based on temperature, then the selection process is likely complicated. Near-surface hydrographic observations (7-17 m depth) within the Southern California Bight indicate that temperature is most variable at the semidiurnal timescale (Frieder et al. 2012). This variability is largely due to K1 and M2 cycles and occurs throughout the bight (Parnell et al. 2006, Frieder et al. 2012). Further, the effects of semidiurnal tides are dampened due to uplift of the isopycnals and increase with depth (Navarro et al. 2013). The environmental factors vary substantially with depth over the embryo habitat (10-90 m depth; Zeidberg et al. 2011, Navarro et al. 2013). For example, when isopycnals are uplifted, long periods of relatively constant low temperature, low pH (high $p\text{CO}_2$), and low $[\text{O}_2]$ bath the upper shelf (Nam et al. 2011, Navarro et al. 2013).

Low pH_{OX} impacts squid-embryo development ability, embryogenesis duration, and statolith size. Low $[\text{O}_2]$ is inferred to be driving these effects. Persistent upwelling events uplift oxyclines along the shelf of the Southern California Bight and similar uplifting occurs during La Niña (Nam et al. 2011). During these times, the shelf is bathed with seawater that is lower in temperature, pH, and $[\text{O}_2]$, with higher levels of $p\text{CO}_2$. We predict that the best embryo habitat in terms of pH, $p\text{CO}_2$ and $[\text{O}_2]$ are within

the upper-shelf waters < 40 m depth with less viable habitat occurring deeper. At shallow depths, semi-diurnal tidal currents bathe embryos with high [O₂] and pH (low pCO₂) waters promoting critical gas exchange (ventilation) during development. *D. opalescens* is predicted to attach embryo capsules at different depths on the shelf throughout the year, based on environmental exposure to [O₂] (see Chapter 2). At depths > 40 m the low pH/O_x conditions are more common and likely cause negative effects on squid embryo development (i.e. these depths are more harsh). The use of the habitat by squid should be investigated to understand whether or not squid site selection varies relative to the environmental conditions, including variability, and whether or not there is evidence to suggest avoidance of low pH/O_x waters.

If squid are utilizing areas that are higher in [O₂] and pH, then there may be a management imperative to conserve this habitat. To sustain a healthy, sustainable fishery and ecosystem services, it may be necessary to protect spawning areas from human impacts including habitat degradation and some forms of fishing. In California, enforcement may be necessary to ensure that squid fishery vessels refrain from scraping the seafloor with purse seine nets (intentional or unintentional) to protect encapsulated embryos from removal and dislodgment.

In addition, squid-embryo habitat is often near areas exposed to persistent nutrient-rich urban runoff in the Southern California Bight. Persistent large-scale eutrophication caused by urban runoff can lead to hypoxic “dead zones” on the shelf (Rabalais et al. 2010, Levin and Dayton 2009). This study shows that encapsulated embryos are negatively affected by near-hypoxic waters. Squid embryo habitat would be

decimated if sustained exposure to hypoxic or even near-hypoxic waters were to occur. If it is not viable to protect coastal areas near urban centers from runoff exposure then other areas should be protected to mitigate for degraded habitat. However appropriate spatial planning cannot be implemented until squid-embryo habitat is mapped over its range as recommended in Chapter 2.

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Figures

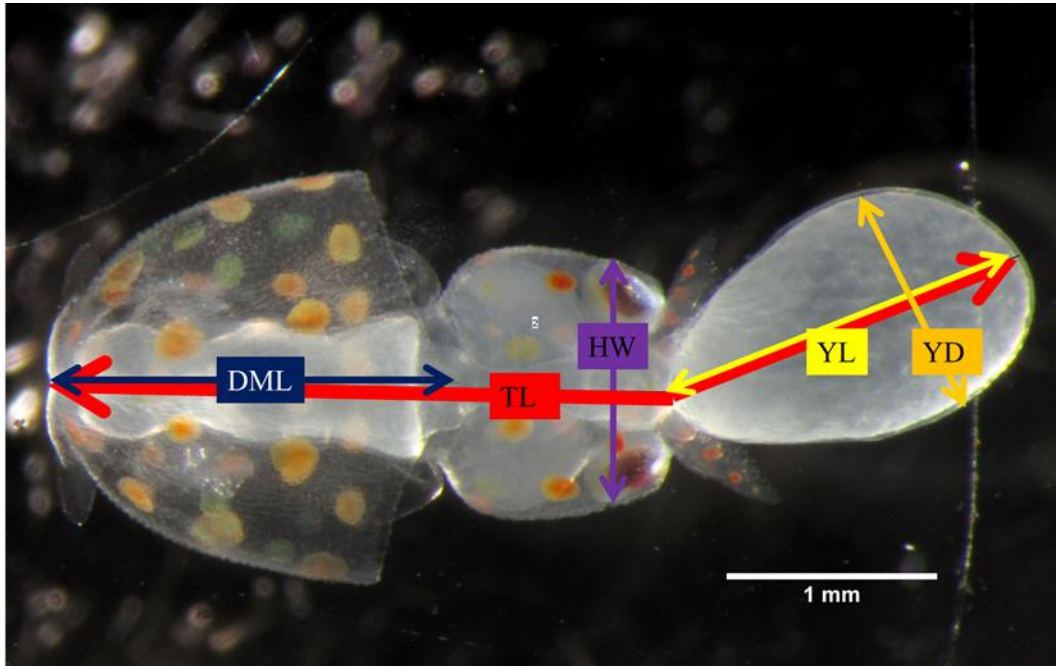


Figure 3.1. Embryo Structures. Dorsal Mantle Length (DML) = Dark blue. Total length (TL) = Red. Head width (HW) = Purple. Yolk length (YL) = Yellow. Yolk diameter (YD) = Orange.

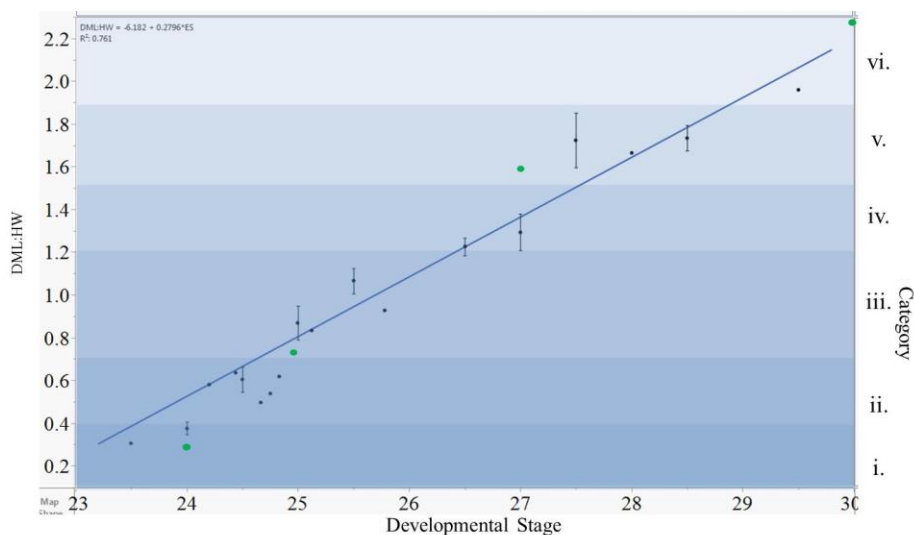


Figure 3.2. Correlation of developmental stages (x-axis) to dorsal mantle length: head width (left y-axis). Developmental category is binned by DML:HW ratios (right y-axis). Blue dots = this study, Green dots = Fields 1965.

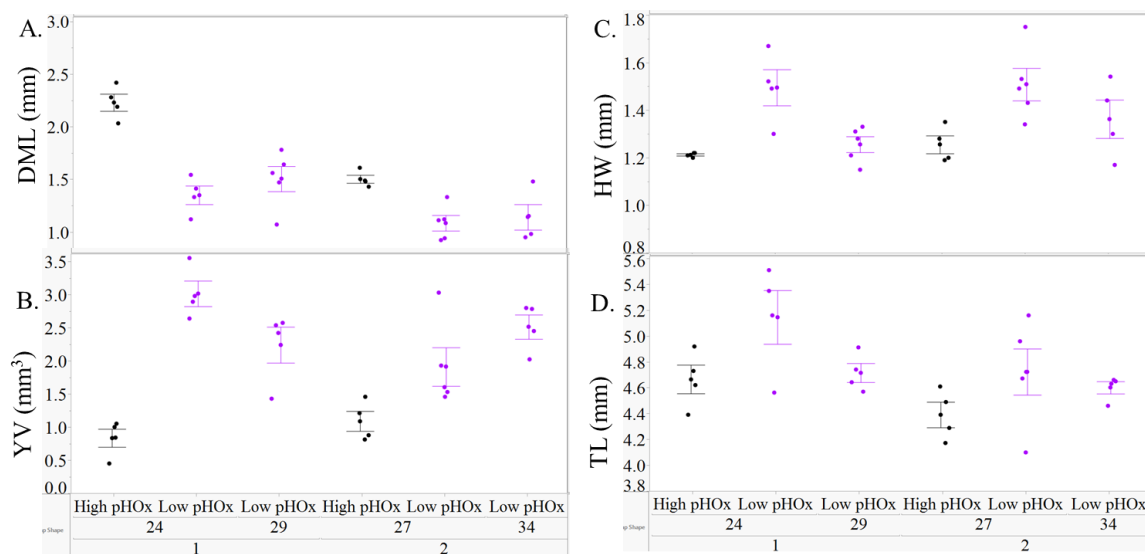


Figure 3.3. Experiment 1. X-axis: Bottom numbers indicate Cohort 1 or 2, middle numbers indicate exposure duration (d) and treatment is indicated by either low pHox (purple) or high pHox (black). A. Y-axis = Dorsal mantle length (mm). B. Y-axis = Yolk volume (mm³). C. Y-axis = Head width (mm). D. Y-axis = Total length (mm). Each point represents the average embryo value per capsule (N= 10 embryos per capsule). * = treatment effect ($P < 0.05$), ** = cohort effect ($P < 0.05$), *** = exposure duration effect ($P < 0.05$). Bars = ± 1 standard error.

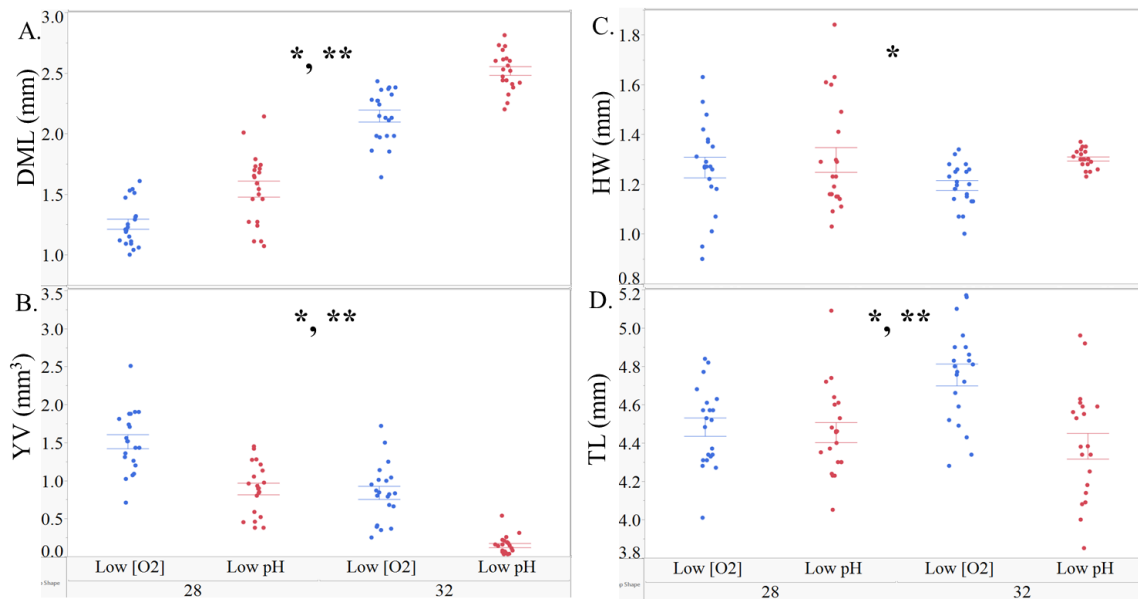


Figure 3.4. Experiment 2. X-axis: Numbers indicate exposure duration (d) and treatment is indicated as either low pH (red) or low [O₂] (blue). A. Y-axis = Dorsal mantle length (mm). B. Y-axis = Yolk volume (mm³). C. Y-axis = Head width (mm). D. Y-axis = Total length (mm). Each point represents the average embryo value per capsule (N= 10 embryos per capsule). * = treatment effect ($P < 0.05$). ** = exposure duration effect ($P < 0.05$). Bars = ± 1 standard error.

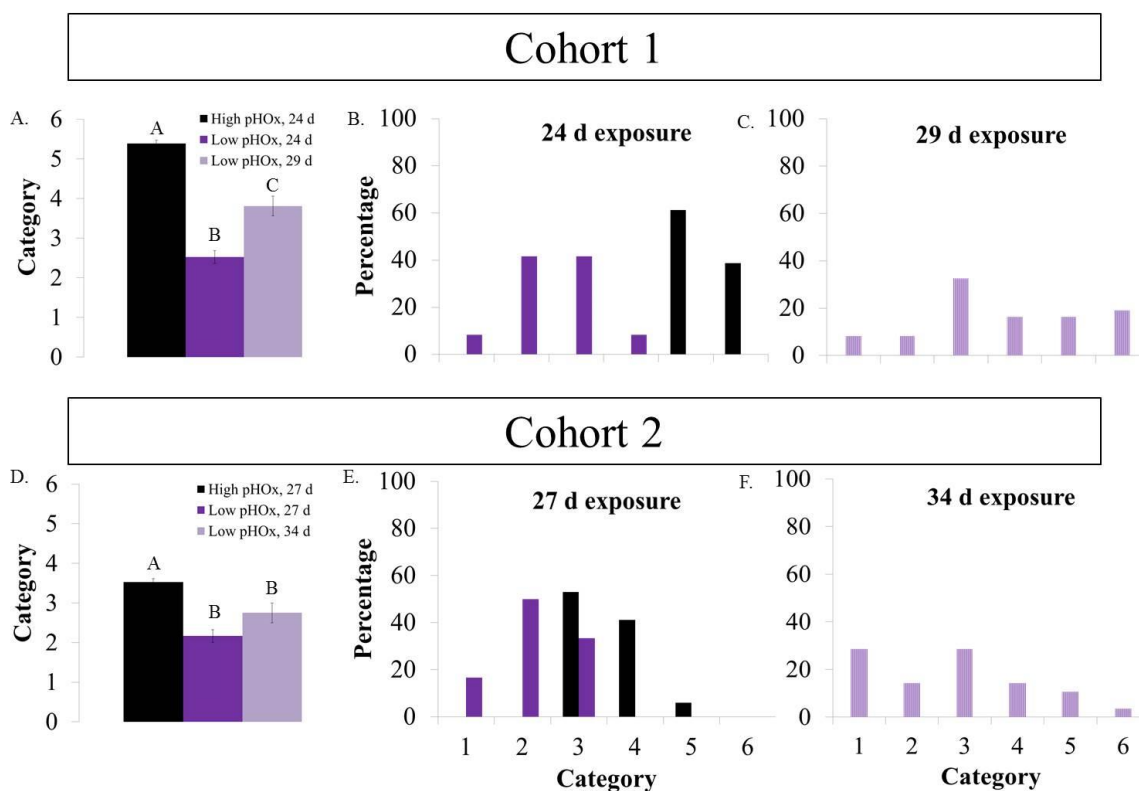


Figure 3.5. Experiment 1. A-C: Cohort 1. A. Developmental categories of embryos removed from the experiment after 24 d of exposure to high pHOx (black), low pHOx treatment (purple). Low pHOx has been separated into two categories; those removed after 24 d of exposure to low pHOx (dark purple) and those removed after 29 d of exposure (light purple). Letters indicate significant difference among groups. Bar = ± 1 standard error. B. Histogram of embryos exposed for 24 d to either high pHOx (black) or low pHOx (dark purple). C. Histogram of embryos exposed for 29 d to low pHOx (light purple). D-F. Cohort 2. D. Developmental categories of embryos removed from the experiment after 27 d of exposure to high pHOx (black) or low pHOx treatment (purple). Exposure to low pHOx was either for 27 d (dark purple) or 34 d (light purple). Letters indicate significant difference among groups. Bar = ± 1 standard error. E. Histogram of embryos exposed for 27 d to either high pHOx (black) or low pHOx (dark purple). F. Histogram of embryos exposed for 34 d to low pHOx (light purple).

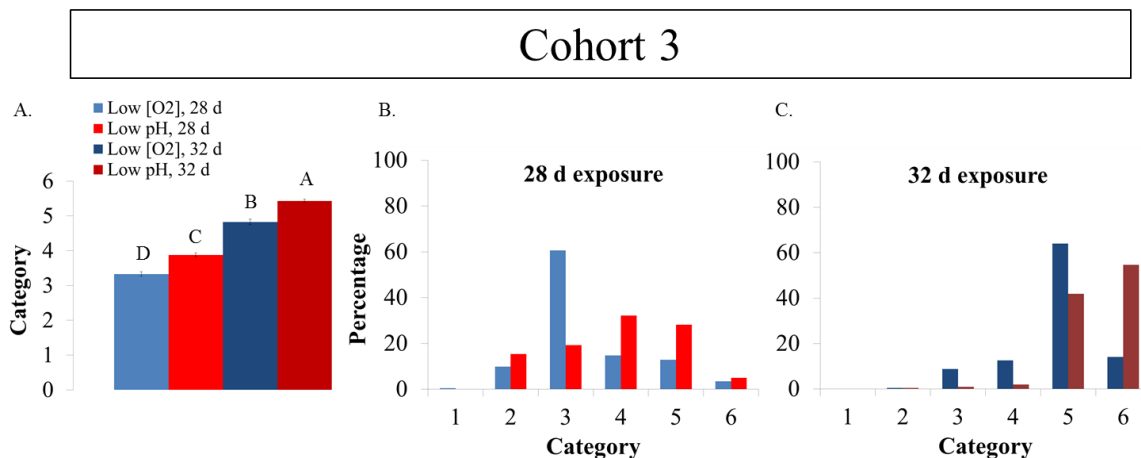


Figure 3.6. Experiment 2, cohort 3. A. Developmental categories of embryos removed from the experiment after 28 d of exposure to low [O₂] (light blue) or low pH (red) and after 32 d of exposure to low [O₂] (dark blue) or low pH (dark red). Letters indicate significant difference among groups. Bar = ± 1 standard error. B. Histogram of embryos removed after 28 d of exposure to low [O₂] (light blue) or low pH (red). C. Histogram of embryos removed after 32 d of exposure to low [O₂] (dark blue) or low pH (dark red).

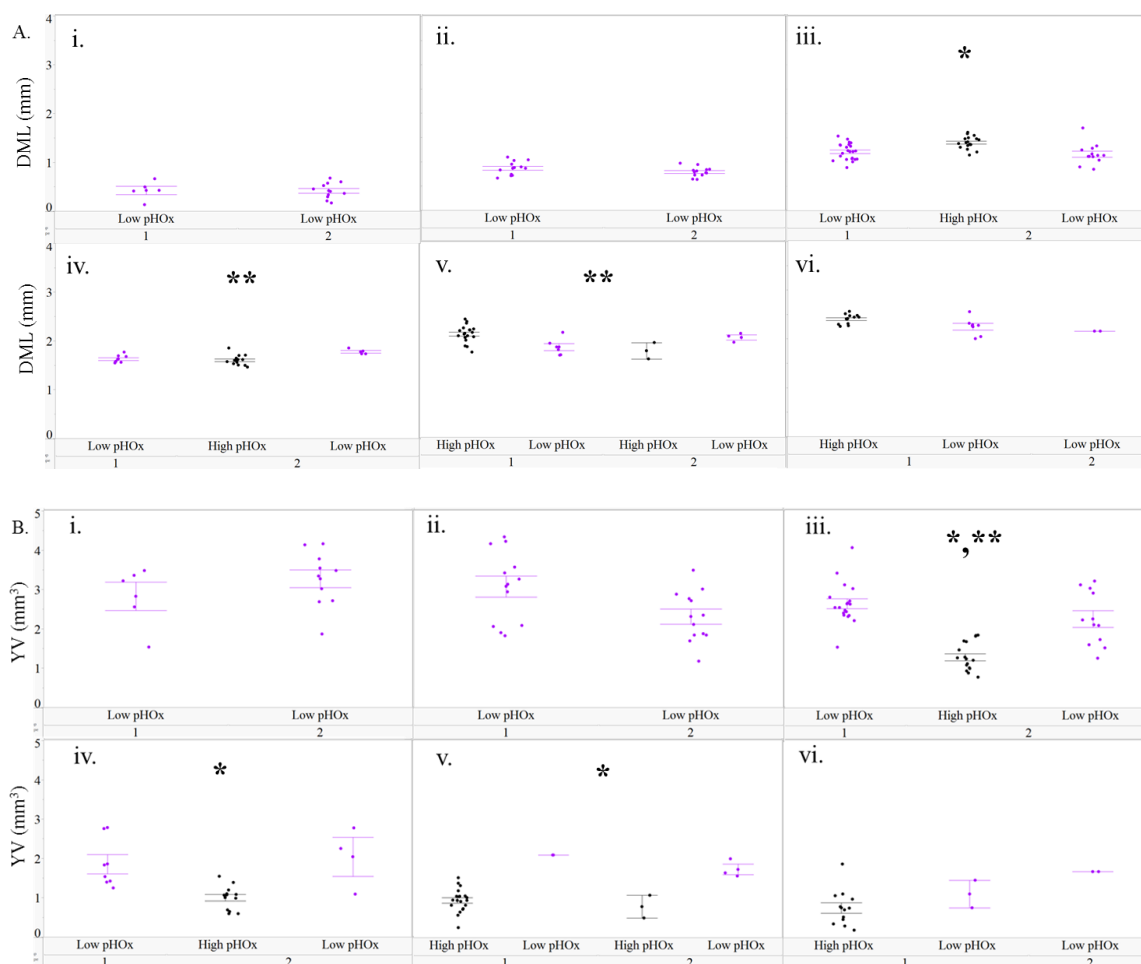


Figure 3.7. Experiment 1 treatment effects on embryonic structures by developmental category. A. Y-axis = Dorsal mantle length (mm). B. Y-Axis = Yolk volume (mm³). i.-vi. = developmental category. X-axis: Numbers indicate cohort. Treatment is either low pHOx (purple) or high pHOx (black). Each point represents the average embryo value per capsule (N= 6.7 embryos per capsule). * = treatment effect ($P < 0.05$). ** = cohort effect ($P < 0.05$). Bars = ± 1 standard error.

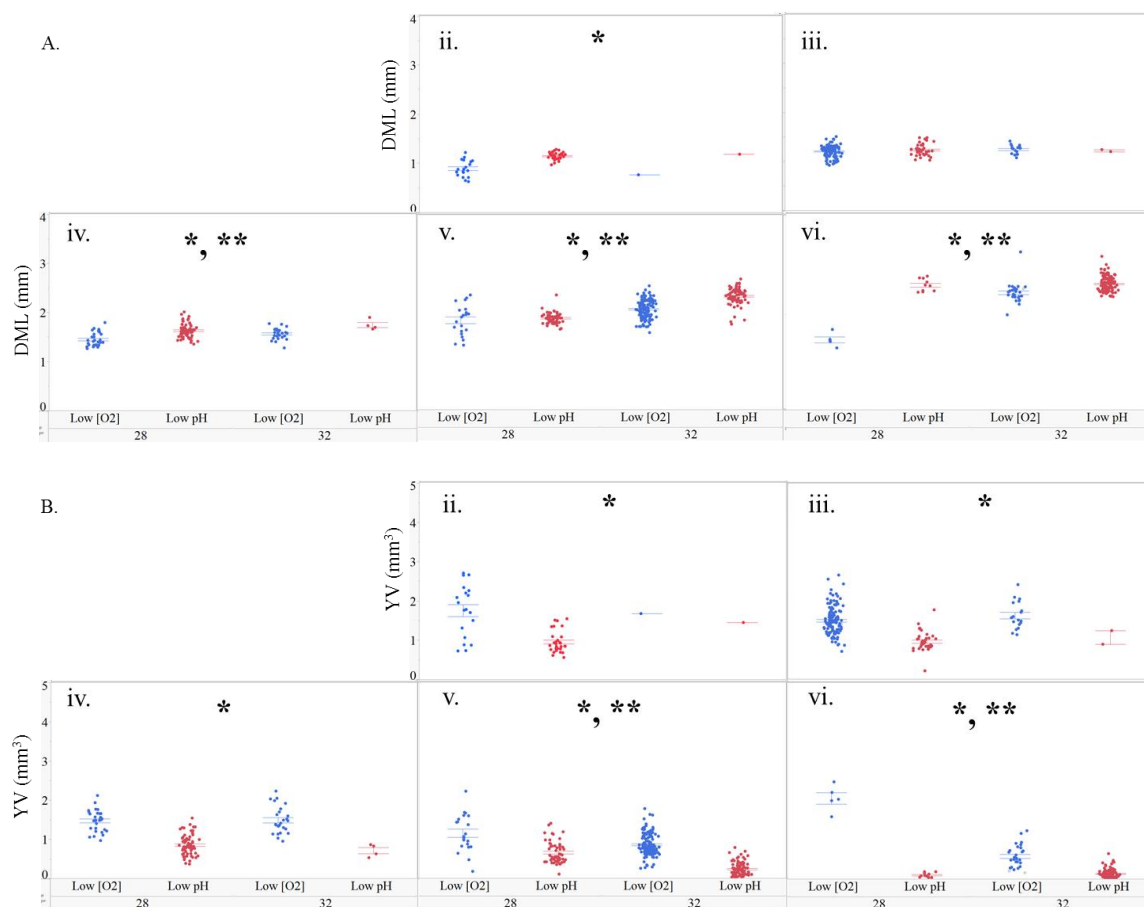


Figure 3.8. Experiment 2 treatment effects on embryonic structures by developmental category. A. Y-axis = Dorsal mantle length (mm). B. Y-axis = Yolk volume (mm³). ii.-vi. = developmental category. X-axis: Numbers indicate cohort. Treatment is indicated either low [O₂] (blue) or low pH (red). Each point represents the average embryo value per capsule (N= 7 embryos per capsule). * = treatment effect ($P < 0.05$). ** = exposure effect ($P < 0.05$). Bars = ± 1 standard error.

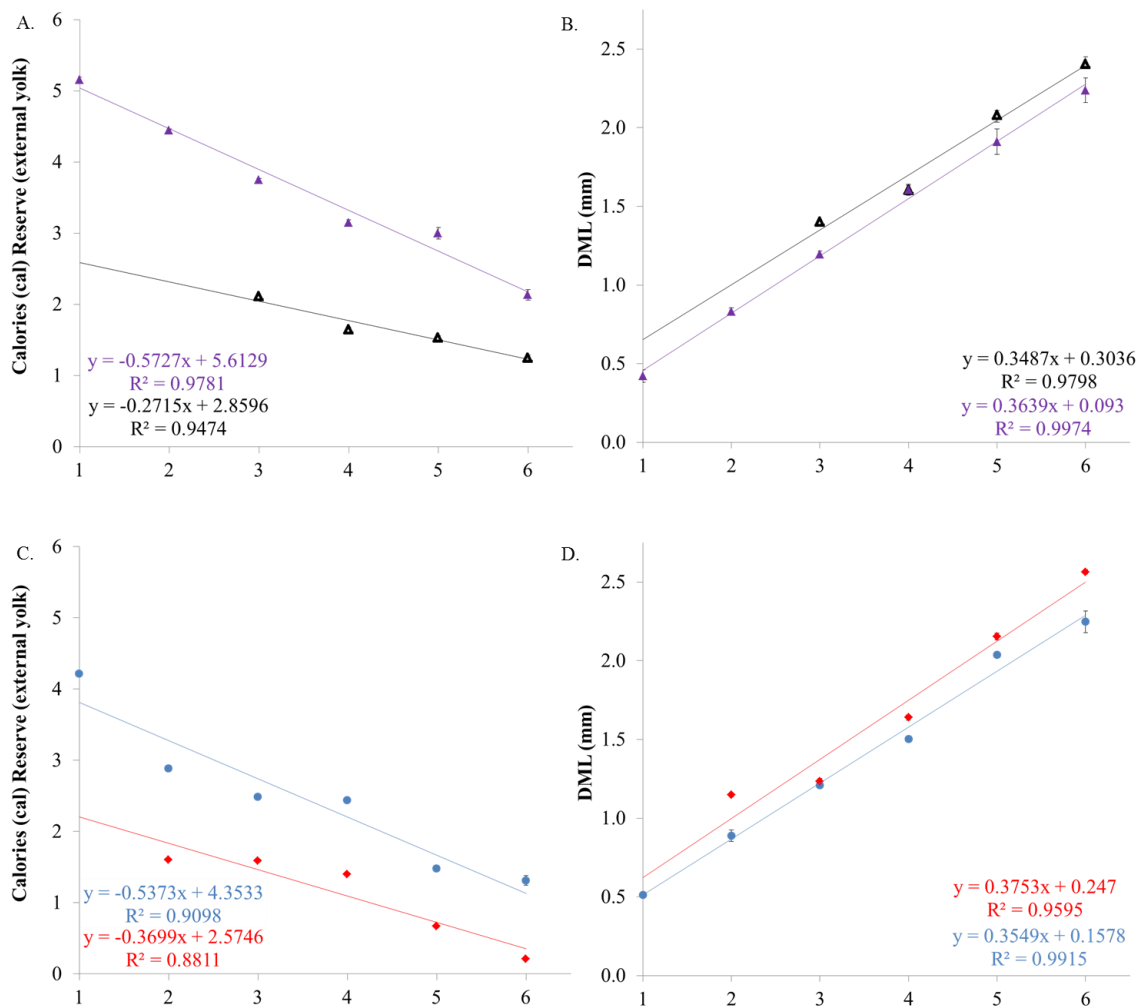


Figure 3.9. Experiment 1: A. Calories (cal) in reserve (external yolk sac; y-axis) per treatment by developmental category (x-axis). B. DML (y-axis) per treatments by developmental category (x-axis). Low pH_{OX} = purple, High pH_{OX} = black. Experiment 2: C. Calories (cal) in reserve (external yolk sac; y-axis) for treatments by developmental category (x-axis). D. DML (y-axis) for treatments by developmental category (x-axis). Low [O₂] = blue, Low pH = red.

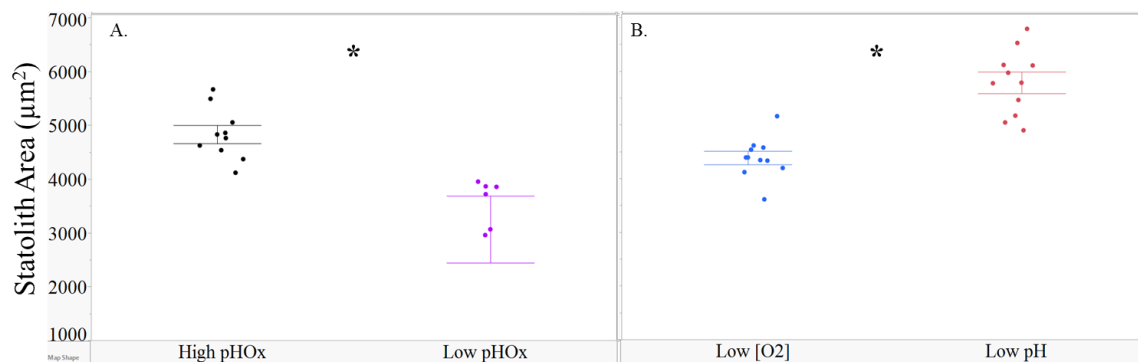


Figure 3.10. Average statolith area (μm^2) per capsule by treatment. Each capsule = Average of five statoliths. A. Experiment 1. All statoliths are from Cohort 1. Low pHOx = purple, High pHOx = black. B. Experiment 2. All statoliths are from the 32-d exposure treatment. Low [O₂] = blue, Low pH = red. Asterisk indicates significant differences between treatment groups. Bars = ± 1 standard error.

Tables

Table 3.1. Developmental categories based on dorsal mantle length: head width. Other columns indicate stages derived from this study and others (Fields 1965, Arnold 1965, and Segawa *et al.* 1988).

Category	DML:HW	This Study	<i>D. opalescens</i> Fields (1965)	<i>D. pealeii</i> Arnold (1965)	<i>L. forbesi</i> Segawa <i>et al.</i> (1988)
1	< 0.4 (Fields = 0.27)	≤ Stage 24	Day 15	Stage 23	Stage 15
2	> 0.4 and < 0.7	-	-	-	-
3	> 0.7 and < 1.2 (Fields = 0.72)	Stage 25	Day 18	Stages 24	Stages 16
4	> 1.2 and < 1.5	Stage 26	-	Stages 25	Stages 17, 18
5	> 1.5 and < 1.9 (Fields = 1.59)	Stage 27-29	Day 21	Stages 26-28	Stages 19-23
6	> 1.9 (Fields = 2.27)	Stage 30, 31	Day 24	Stages 29-30	Stages 24-27

Table 3.2. Near-hatch paralarva (category 6) caloric reserve and size (DML) by treatment.

	Low pHOx	High pHOx	Low [O ₂]	Low pH
Unused reserve (cal)	2.138 cal	1.249 cal	1.312 cal	0.215 cal
Size (mm)	2.238 mm	2.405 mm	2.248 mm	2.565 mm

Appendix Figures and Tables

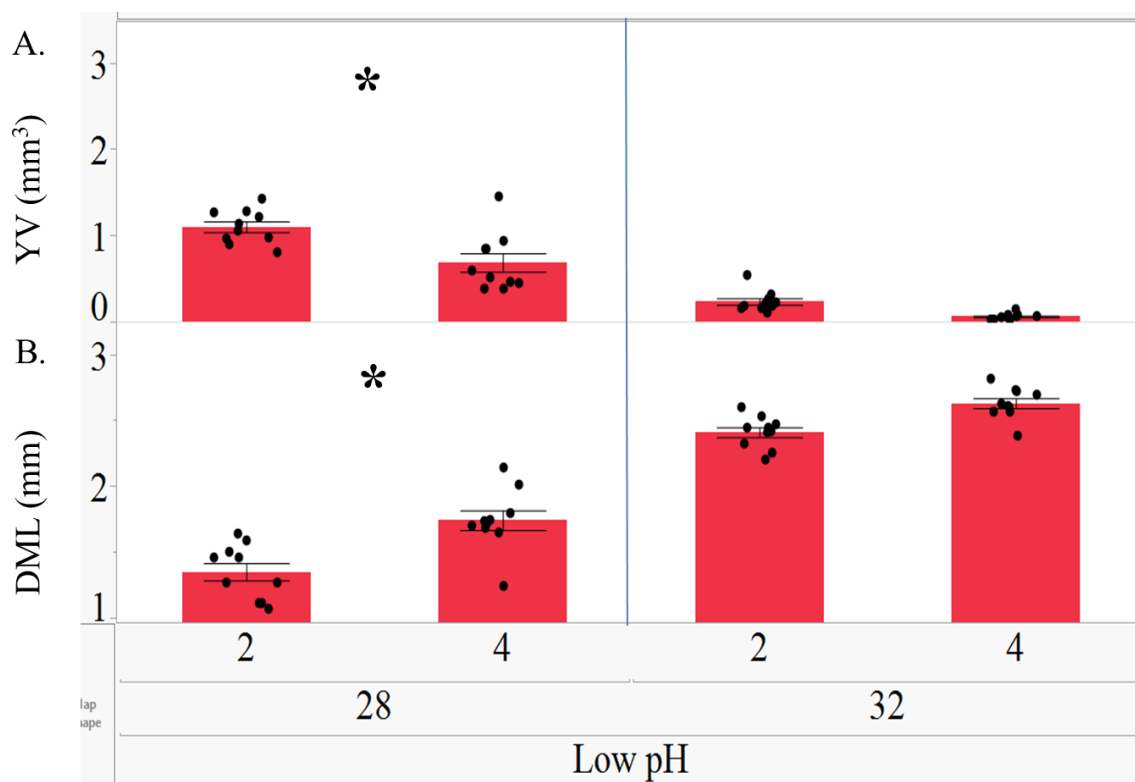


Figure 3.A1. Tank effects. X-Axis = Low pH treatment after 28 and 32 days of exposure, within tank #2 or #4. A. Yolk volume and B. dorsal mantle length. Both differed between embryos in tank #2 and #4 when exposed to low pH for 28 days but did not differ when exposed for 32 days. Bar = ± 1 standard error.

Table 3.A1. Treatment effects across developmental categories. A. Experiment 1, treatments = low pH_{Ox}, high pH_{Ox}. B. Experiment 2, treatments = low pH, low [O₂]. Cat = Embryonic-squid developmental category, DML = dorsal mantle length, YV = external yolk sac volume, HW = head width, TL = total length of the embryo and external yolk sac. Bold and italicized font = significant, NS = non-significant.

A.

Cat	DML	YV	HW	TL
2	NS	NS	NS	NS
3	<i>$F_{1,51} = 13.587,$ $P = 0.0025$</i>	<i>$F_{1,51} = 21.745,$ $P = 0.0009$</i>	NS	NS
4	NS	<i>$F_{1,25} = 7.389,$ $P = 0.0413$</i>	NS	<i>$F_{1,22} = 14.662,$ $P = 0.0111$</i>
5	NS	<i>$F_{1,29} = 13.961,$ $P = 0.0027$</i>	NS	NS
6	<i>$F_{1,19} = 5.791,$ $P = 0.058$</i>	NS	<i>$F_{1,19} = 4.972,$ $P = 0.0752$</i>	NS

B.

Cat	DML	YV	HW	TL
2	<i>$F_{1,51} = 15.139,$ $P = 0.0016$</i>	<i>$F_{1,51} = 14.432,$ $P = 0.0018$</i>	<i>$F_{1,51} = 9.154,$ $P = 0.0087$</i>	NS
3	NS	<i>$F_{1,177} = 18.717,$ $P < 0.0001$</i>	<i>$F_{1,177} = 8.633,$ $P = 0.0041$</i>	NS
4	<i>$F_{1,124} = 18.568,$ $P < 0.0001$</i>	<i>$F_{1,124} = 54.282,$ $P < 0.0001$</i>	<i>$F_{1,124} = 8.944,$ $P = 0.0038$</i>	NS
5	<i>$F_{1,295} = 13.315,$ $P = 0.0004$</i>	<i>$F_{1,295} = 28.965,$ $P < 0.0001$</i>	<i>$F_{1,295} = 31.202,$ $P < 0.0001$</i>	NS
6	<i>$F_{1,1} = 167.293,$ $P < 0.0001$</i>	<i>$F_{1,1} = 116.252,$ $P < 0.0001$</i>	<i>$F_{1,1} = 286.099,$ $P < 0.0001$</i>	NS

Table 3.A2. Cohort effects across developmental categories for Experiment 1. Cat = Embryonic-squid developmental category, DML = dorsal mantle length, YV = external yolk sac volume, HW = head width, TL = total length of the embryo and external yolk sac. Bold and italicized font = significant, NS = non-significant.

Cat	DML	YV	HW	TL
2	NS	NS	NS	NS
3	NS	NS	$F_{2,51} = 4.898,$ $P = 0.0258$	$F_{2,45} = 4.965,$ $P = 0.0461$
4	$F_{2,25} = 6.696,$ $P = 0.0218$	NS	NS	NS
5	$F_{2,29} = 4.553,$ $P = 0.0352$	NS	NS	NS
6	NS	NS	NS	NS

Table 3.A3. Effects of time-of-exposure across developmental categories. A. Experiment 1 (low pH0x treatment only), Cohort 1 time of exposure = 24 d, 29 d, Cohort 2 time of exposure = 27 d, 34 d. B. Experiment 2, time of exposure = 28 d, 32 d. Cat = Embryonic-squid developmental category, DML = dorsal mantle length, YV = external yolk sac volume, HW = head width, TL = total length of the embryo and external yolk sac. Bold and italicized font = significant, NS = non-significant.

A.

Cat	DML	YV	HW	TL
2	NS	NS	NS	$F_{4,23} = 7.5133,$ $P = 0.0039$
3	NS	$F_{4,17} = 4.898,$ $P = 0.0258$	NS	NS
4	NS	NS	NS	NS

B.

Cat	DML	YV	HW	TL
2	NS	NS	NS	NS
3	NS	NS	NS	NS
4	$F_{4,124} = 3.517,$ $P = 0.0363;$	NS	NS	$F_{4,124} = 3.536,$ $P = 0.0345$
5	$F_{4,295} = 81.251,$ $P < 0.0001;$	$F_{4,295} = 28.965,$ $P < 0.0001$	$F_{4,295} = 33.946,$ $P < 0.0001$	NS
6	$F_{4,154} = 60.492,$ $P < 0.0001$	$F_{4,154} = 116.252,$ $P < 0.0001$	$F_{4,154} = 112.903,$ $P < 0.0001$	$F_{4,154} = 16.669,$ $P < 0.0001$

Table 3.A4. Capsule effects across developmental categories. A. Experiment 1. B. Experiment 2. Cat = Embryonic-squid developmental category, DML = dorsal mantle length, YV = external yolk sac volume, HW = head width, TL = total length of the embryo and external yolk sac. Bold and italicized font = significant, NS = non-significant.

A.

Cat	DML	YV	HW	TL
2	NS	NS	NS	NS
3	NS	$F_{8,51} = 2.560$, $P = 0.0242$	NS	$F_{8,45} = 3.944$, $P = 0.0018$
4	NS	$F_{8,25} = 13.593$, $P = 0.0001$	$F_{8,25} = 4.287$, $P = 0.0116$	$F_{8,25} = 3.334$, $P = 0.0406$
5	$F_{8,29} = 2.717$, $P = 0.0464$	NS	$F_{8,29} = 3.445$, $P = 0.0192$	$F_{8,29} = 9.721$, $P = 0.0004$
6	NS	$F_{8,14} = 5.424$, $P = 0.0209$	NS	NS

B.

Cat	DML	YV	HW	TL
2	$F_{72,51} = 8.199$, $P < 0.0001$	$F_{72,51} = 7.383$, $P < 0.0001$	$F_{72,51} = 7.026$, $P < 0.0001$	$F_{72,51} = 5.441$, $P = 0.0001$
3	$F_{72,177} = 2.139$, $P = 0.0009$	$F_{72,177} = 4.568$, $P < 0.0001$	$F_{72,177} = 3.088$, $P < 0.0001$	$F_{72,177} = 3.084$, $P < 0.0001$
4	$F_{72,124} = 5.928$, $P < 0.0001$	$F_{72,124} = 5.445$, $P < 0.0001$	$F_{72,124} = 2.973$, $P < 0.0001$	$F_{72,124} = 3.085$, $P < 0.0001$
5	$F_{72,295} = 1.867$, $P = 0.0016$	$F_{72,295} = 5.211$, $P < 0.0001$	$F_{72,295} = 2.463$, $P < 0.0001$	$F_{72,295} = 3.750$, $P < 0.0001$
6	$F_{72,154} = 3.272$, $P < 0.0001$	$F_{72,154} = 8.873$, $P < 0.0001$	$F_{72,154} = 2.511$, $P < 0.0001$	$F_{72,154} = 4.448$, $P < 0.0001$

CHAPTER 4.

Environmental pH, O₂ and Capsular Effects on the Geochemical Composition of Statoliths of Embryonic Squid *Doryteuthis opalescens*

Synopsis

This chapter is the companion paper to Chapter 3 using the same laboratory experiments. Statoliths of the embryos developed in these experiments were analyzed geochemically. This chapter is presented as a paper. “Environmental pH, O₂ and capsular effects on the geochemical composition of statoliths of embryonic squid *Doryteuthis opalescens*,” published in Water (2014), explores the potential for use of statoliths as geochemical proxies for environmental pH and O₂ exposure.

Article

Environmental pH, O₂ 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 [O₂]. 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 [O₂] observed on squid-embryo beds to test in the laboratory whether or not squid statolith geochemistry reflects environmental pH and [O₂]. We asked whether pH and [O₂] 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 [O₂] exposure. Embryo exposure to high and low pH and [O₂] 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 $[\text{O}_2]$ (79–82 $\mu\text{mol}\cdot\text{kg}^{-1}$) than those exposed to higher ambient pH_T (7.92–7.94) and $[\text{O}_2]$ (241–243 $\mu\text{mol}\cdot\text{kg}^{-1}$). In a separate experiment, exposure to low pH_T (7.55–7.56) or low $[\text{O}_2]$ (83–86 $\mu\text{mol}\cdot\text{kg}^{-1}$) yielded elevated U:Ca and Sr:Ca in the low $[\text{O}_2]$ 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 $[\text{O}_2]$). We show that statoliths of squid embryos developing inside capsules have the potential to reflect environmental pH and $[\text{O}_2]$, 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 $[\text{O}_2]$ through space and time [1–7]. Environmental fluctuations in seawater properties, including low levels of pH and $[\text{O}_2]$, can cause sublethal and lethal effects in loliginids (nearshore squid; [8]). Within squid habitats, low pH and $[\text{O}_2]$ seawater can be tightly associated with upwelling events [2]. Average pH and $[\text{O}_2]$ 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 $p\text{CO}_2$ 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 $[\text{O}_2]$ 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 $[\text{O}_2]$ and pH/ $p\text{CO}_2$ 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 $\delta^{11}B$ 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 $p\text{CO}_2$ and low $[\text{O}_2]$ (hereafter referred to as “low pH/Ox”) 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 pH/Ox, low $[\text{O}_2]$ only and/or low pH/high $p\text{CO}_2$ 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 pH/Ox 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 pH/Ox levels yields elemental signatures different from individual effects of low $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $[\text{O}_2]$ (high pH/Ox) and low pH and $[\text{O}_2]$ (low pH/Ox) treatments, and Experiment 2 (March, 2012) compared the statolith geochemical response between embryos exposed to low $[\text{O}_2]$ and high pH (low $[\text{O}_2]$) and low pH and high $[\text{O}_2]$ (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 $[\text{O}_2]$) decreases with depth. For example, the range of $[\text{O}_2]$ decreased by 37% and the range of pH by 39% from a 7 to 17-m depth [3]. The variability $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $\mu\text{mol}\cdot\text{kg}^{-1}$) and high levels (7.9, 240 $\mu\text{mol}\cdot\text{kg}^{-1}$) 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 , $\Omega_{\text{aragonite}}$ and $[\text{O}_2]$, 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 $>18.0 \text{ M}\Omega\cdot\text{cm}$). Treatments were implemented using MSEAS, a manipulated flow-through aquarium design [67]. Seawater was supplied with seawater pumped from a $5 \pm 1.5 \text{ m}$ depth off of the Scripps Pier.

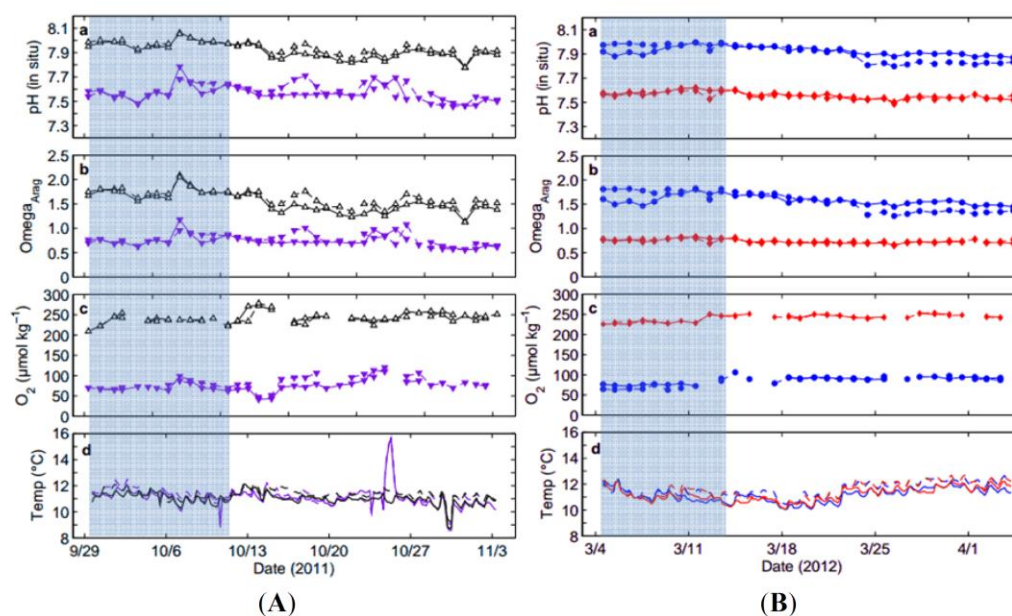
Table 1. Average ± 1 standard deviation for temperature ($^{\circ}\text{C}$), alkalinity ($\mu\text{mol}\cdot\text{kg}^{-1}$), pH_T , and $[\text{O}_2]$ ($\mu\text{mol}\cdot\text{kg}^{-1}$) in tanks during Experiment 1 and Experiment 2. Treatments were distinct for pH_T , $\Omega_{\text{aragonite}}$ and $[\text{O}_2]$, but not temperature and alkalinity (Wilcoxon test).

Treatment (Tank)	Temp ($^{\circ}\text{C}$)	Alkalinity ($\mu\text{mol}\cdot\text{kg}^{-1}$)	pH_T (in-situ) Total Scale	$\Omega_{\text{Aragonite}}$	$[\text{O}_2]$ ($\mu\text{mol}\cdot\text{kg}^{-1}$)
Experiment 1					
High pHOx (1)	11.3 \pm 0.4	2215.5 \pm 4.8	7.938 \pm 0.053	1.62 \pm 0.17	241.3 \pm 12.3
High pHOx (2)	11.1 \pm 0.4	2214.2 \pm 6.6	7.916 \pm 0.062	1.54 \pm 0.21	242.6 \pm 13.1
Low pHOx (1)	11.4 \pm 0.8	2214.8 \pm 6.3	7.578 \pm 0.067	0.76 \pm 0.12	82.1 \pm 15.8
Low pHOx (2)	11.2 \pm 0.9	2215.4 \pm 5.8	7.567 \pm 0.065	0.74 \pm 0.12	78.6 \pm 21.5
Treatment Effect (df = 1, N = 36)	$\chi^2 = 0.02$, $p = 0.876$	$\chi^2 = 0.01$, $p = 0.921$	$\chi^2 = 109.35$ $p < 0.0001$	$\chi^2 = 109.35$ $p < 0.0001$	$\chi^2 = 90.76$, $p < 0.0001$
Experiment 2					
Low $[\text{O}_2]$ (1)	11.2 \pm 0.5	2239.1 \pm 5.5	7.923 \pm 0.035	1.58 \pm 0.10	86.4 \pm 8.3
Low $[\text{O}_2]$ (2)	11.6 \pm 0.5	2241.8 \pm 4.5	7.908 \pm 0.072	1.57 \pm 0.21	83.0 \pm 12.9
Low pH (1)	11.3 \pm 0.5	2241.1 \pm 5.8	7.559 \pm 0.029	0.73 \pm 0.04	241.1 \pm 9.1
Low pH (2)	11.6 \pm 0.6	2244.2 \pm 7.1	7.552 \pm 0.026	0.73 \pm 0.04	241.7 \pm 7.6
Treatment Effect (df = 1, N = 32)	$\chi^2 = 0.05$, $p = 0.819$	$\chi^2 = 3.14$, $p = 0.077$	$\chi^2 = 93.74$, $p < 0.0001$	$\chi^2 = 93.74$, $p < 0.0001$	$\chi^2 = 72.74$, $p < 0.0001$

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 $^{\circ}\text{C}$. 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 $\cdot\text{m}^{-2}$) and 2 (density ~ 200 capsules $\cdot\text{m}^{-2}$) are within the viable range found in the field, where they are reported to range from 1 to 47,720 capsules $\cdot\text{m}^{-2}$ [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 ($\Omega_{\text{aragonite}}$), (c) $[\text{O}_2]$ ($\mu\text{mol}\cdot\text{kg}^{-1}$) and (d) temperature ($^{\circ}\text{C}$) of the seawater within each tank of (A) Experiment 1 and (B) Experiment 2. Purple = Low pHOx; Black = High pHOx; Blue = Low $[\text{O}_2]$; 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).

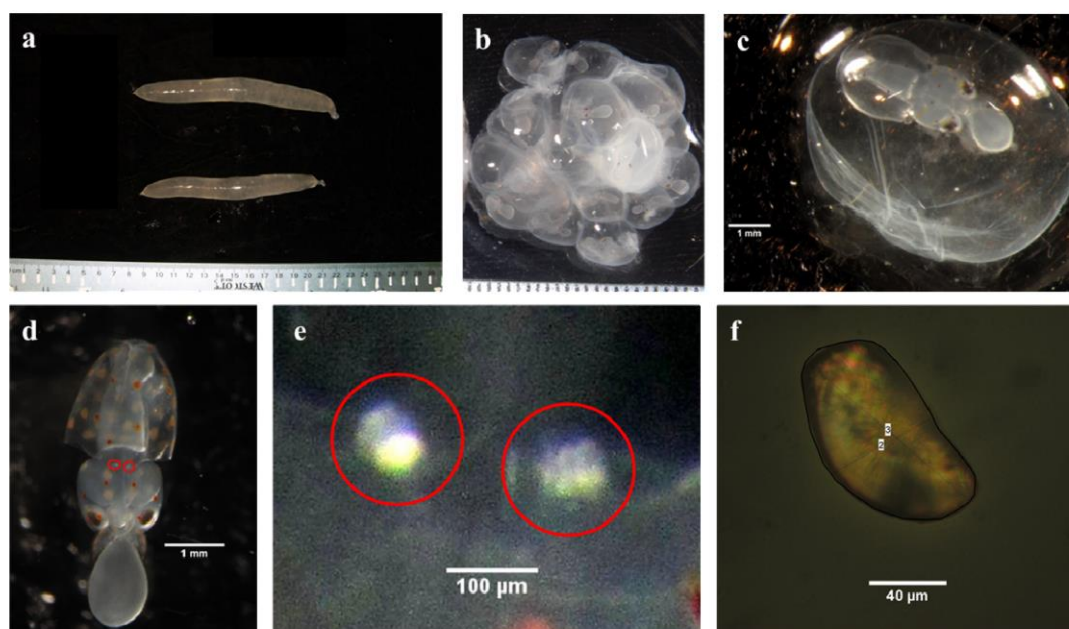


Embryos were cultured to embryonic-developmental Stages 28 or 29 (near-hatch paralarvae) [68] to reduce ontogenetic effects [71] and were collected only within the central portion of the capsule in order to reduce capsule-position effects [72] (Figure 2). These stages were indicated by the pigmentation 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 ($11.3\text{ }^{\circ}\text{C} \pm 0.3\text{ }^{\circ}\text{C}$, 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 $[\text{O}_2]$ 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 $[\text{O}_2]$) 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, $\Omega_{\text{Aragonite}}$ 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 ($21\text{ }^\circ\text{C} \pm 3\text{ }^\circ\text{C}$) until they were analyzed at Arizona State University for magnesium²⁴(Mg), calcium⁴⁸(Ca), strontium⁸⁸(Sr), barium¹³⁸(Ba) and uranium²⁸(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 H₂O. All remaining H₂O 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™), 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 boron¹¹ (B), magnesium²⁴ (Mg), calcium⁴⁸ (Ca), manganese⁵⁵ (Mn), copper⁶³ (Cu), zinc⁶⁶ (Zn), strontium⁸⁸ (Sr), barium¹³⁸ (Ba), lead²⁰⁸ (Pb) and uranium²³⁸ (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 μL·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% HNO₃ during analysis of standards, on instrument blanks and on laser-ablated samples [75]. Instrument sensitivity was monitored measuring indium (In) and was approximately 1 × 10⁶ counts·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% CaCO₃ [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 CaCO₃ 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 ($\mu\text{mol:mol}$) = 0.18%, Pb:Ca ($\mu\text{mol:mol}$) = 11.13%, U:Ca ($\mu\text{mol}\cdot\text{mol}^{-1}\text{mol}$) = 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 [O₂] 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).

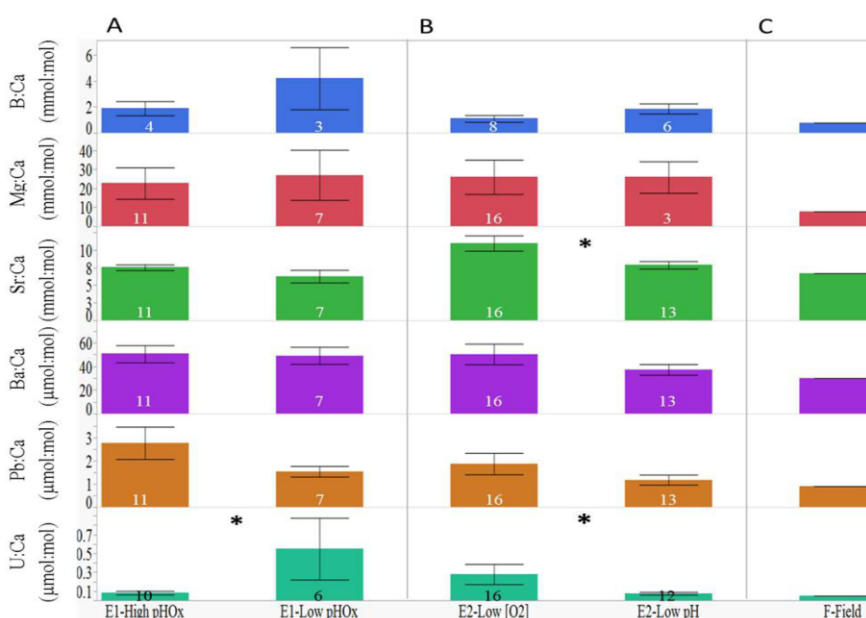
3. Results and Discussion

Seawater element concentrations were not different among treatments in Experiment 1 or Experiment 2 (Appendix Table A1). Further, chronic exposure to undersaturated seawater ($\Omega_{\text{aragonite}} < 1$; 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 $\mu\text{mol}/\text{mol}$). 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 $[\text{O}_2]$ treatments induced a similar or distinct response. In this experiment, the low $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $[\text{O}_2]$ 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-[O₂] 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 [O₂] 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	$F_{3,4} = 6.14$, $p < \mathbf{0.0001}$	$F_{6,10} = 17.43$, $p < \mathbf{0.0001}$
Mg:Ca	$F_{3,14} = 20.15$, $p < \mathbf{0.0001}$	$F_{6,25} = 45.07$, $p < \mathbf{0.0001}$
Sr:Ca	$F_{3,14} = 13.27$, $p < \mathbf{0.0001}$	$F_{6,25} = 41.74$, $p < \mathbf{0.0001}$
Ba:Ca	$F_{3,14} = 2.82$, $p = \mathbf{0.0026}$	$F_{6,25} = 51.12$, $p < \mathbf{0.0001}$
Pb:Ca	$F_{3,14} = 31.05$, $p < \mathbf{0.0001}$	$F_{6,25} = 39.92$, $p < \mathbf{0.0001}$
U:Ca	$F_{3,13} = 2.65$, $p = \mathbf{0.0060}$	$F_{6,25} = 103.28$, $p < \mathbf{0.0001}$

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 (⁷⁵Se, ⁶⁵Zn) and one non-essential element (^{110m}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 [O₂] and *p*CO₂ [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.

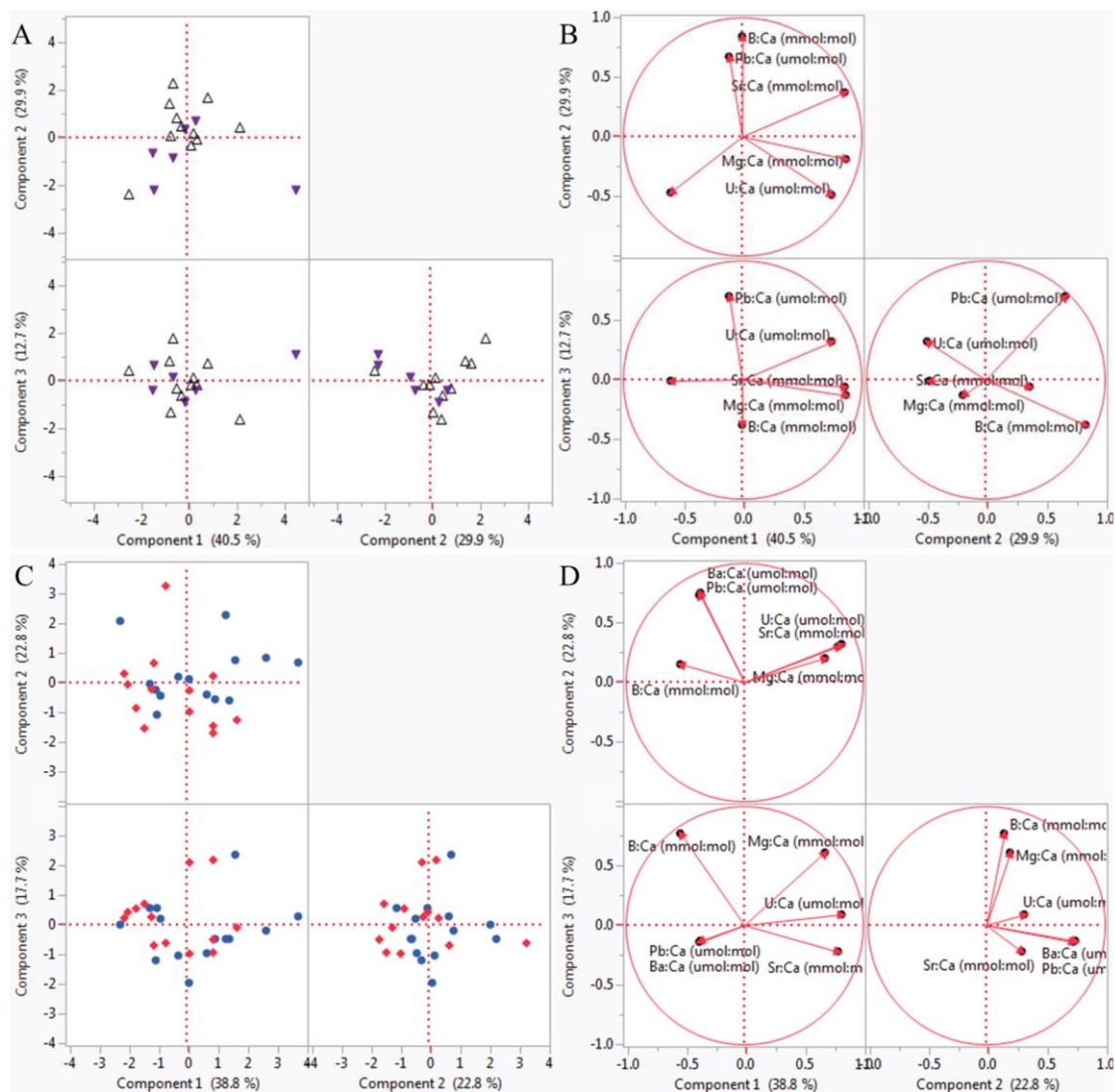
Table 3. Statolith elemental composition differences among capsules within each treatment were tested using a one-way analysis of similarities (Euclidean-distance matrix; elemental menu = B:Ca, Mg:Ca, Sr:Ca, Ba:Ca, U:Ca; $N = 9999$ permutations for all treatments, except low pHox ($N = 3$)).

Groups	R Statistic	<i>p</i> -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 (μ mol:mol)	-0.38689	-0.35043	0.37102	0.78068	-0.04598
Pb:Ca (μ mol:mol)	-0.06976	0.50151	0.80384	0.24169	-0.15971
U:Ca (μ mol: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 (μ mol:mol)	-0.24548	0.64466	-0.12698	0.66566	-0.15275
Pb:Ca (μ mol:mol)	-0.24588	0.62852	-0.13803	-0.71931	0.08053
U:Ca (μ mol: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 pH_{OX}; Black = high pH_{OX}. 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 [O₂]; Red = low pH.



3.3. Statoliths as an Indicator of Environmental Response

The experiments conducted here provide the first evidence that embryonic statolith geochemistry

(241–242 $\mu\text{mol}\cdot\text{kg}^{-1}$), 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 $[\text{CO}_3^{2-}]$ ([61,62,85]). Because we did not find evidence of U enrichment within statoliths in the low-pH treatment, we presume that $[\text{CO}_3^{2-}]$ is also regulated within endolymph fluid.

These results suggest that both pH and $[\text{CO}_3^{2-}]$ 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 $p\text{CO}_2$ /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 $p\text{CO}_2$ /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 $p\text{CO}_2$ /low pH conditions and the compensatory mechanisms that are involved.

Development of an environmental $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $[\text{O}_2]$ 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 $p\text{CO}_2$ 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 $[\text{O}_2]$ and exacerbated by the interactive effect of low $[\text{O}_2]$ and pH (low pHOx). Under low pH stress, low $[\text{O}_2]$ 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 of, we demonstrated that environmental pH and [O₂] 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 ⁶⁵Zn significantly differed from an elemental suite that included ^{110m}Ag, ¹⁰⁹Cd, ⁵⁷Co, ²⁰³Hg and ⁵⁴Mn [49]. Evidence that environmental tracers in squid statoliths can track seawater pH and [O₂] 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 [O₂] effects can be resolved for the use of statolith geochemistry as environmental proxies of pH and [O₂]. 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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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 \pm 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 pH _{OX} (1)	4.45 \pm 0.005	1092 \pm 11.6	351.0 \pm 3.40	5.64 \pm 0.055	4.41 \pm 0.120	1.98 \pm 0.410
Low pH _{OX} (2)	4.45 \pm 0.005	1100 \pm 14.8	353.2 \pm 4.39	5.70 \pm 0.084	4.51 \pm 0.129	2.03 \pm 0.408
High pH _{OX} (1)	4.45 \pm 0.003	1113 \pm 12.6	357.7 \pm 4.16	5.76 \pm 0.064	4.65 \pm 0.062	2.12 \pm 0.428
High pH _{OX} (2)	4.44 \pm 0.014	1118 \pm 14.7	358.5 \pm 5.00	5.78 \pm 0.074	4.51 \pm 0.153	2.03 \pm 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 \pm 0.004	1100 \pm 14.9	355.6 \pm 4.99	5.69 \pm 0.077	4.34 \pm 0.115	2.11 \pm 0.190
Low [O ₂] (2)	4.48 \pm 0.004	1115 \pm 15.1	358.6 \pm 6.28	5.76 \pm 0.089	4.51 \pm 0.168	2.20 \pm 0.176
Low pH (1)	4.48 \pm 0.003	1131 \pm 17.8	365.4 \pm 6.10	5.87 \pm 0.097	4.41 \pm 0.067	2.08 \pm 0.247
Low pH (2)	4.48 \pm 0.004	1108 \pm 31.7	359.2 \pm 10.45	5.72 \pm 0.163	4.34 \pm 0.083	1.85 \pm 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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CHAPTER 5.

Conclusions

This dissertation examined the effects of the environment on squid spawning site selection and on embryogenesis of *D. opalescens*. The environment of the embryo beds was shown to be dynamic with highly variable temperature, pH, O₂ and salinity at semidiurnal and diurnal frequencies (Chapter 2). ROV surveys of embryo beds on the La Jolla shelf revealed that when the temperature, pH, and O₂ were high and salinity was low, the embryo bed was diffuse, covering a large area. In contrast, the embryo bed was condensed, covering a small area when the temperature, pH and O₂ were low (and salinity was high; Chapter 2). This pattern provides key insight into habitat selection throughout the year. The shelf is not always utilized the same way. However, spawning did occur continuously throughout the year and the core area where embryos were consistently laid was in the shallowest portion of the available embryo habitat (20-40 m depth).

The laboratory studies discussed in Chapters 3 and 4 showed that low pHOx (pH and O₂) can slow and negatively affect embryo development. In nature, the lowest pHOx occurs at increased depth. Low pHOx conditions also are correlated with several temporal scales and cycles. They occur during upwelling events, during spring and summer seasons, and during La Niña. The chronic exposure conditions used in the laboratory experiment described in Chapters 3 and 4 best match seasonal and/or ENSO cycles, as well as those in deeper waters (but not 5-7 d upwelling events). Based on findings from the lab I propose that the deepest portions of embryo beds during summer

and/or La Niña, are negatively impacted by low pH_{Ox}. This hypothesis needs testing in the field.

Temperature strongly affects *D. opalescens* embryogenesis (Zeidberg et al. 2011) and temperature correlates strongly with pH and oxygen conditions. Since temperature was not manipulated in this laboratory study, combined effects of temperature and pH and oxygen are still unknown. However, since low temperature also slows development, further laboratory studies are needed to determine whether or not the temperature and pH and oxygen significantly interact in their influence on squid embryogenesis. For example, if these factors interact, is the interaction additive, multiplicative or some other type of relationship? Further, in the laboratory setting squid capsules should be tested to evaluate effects of exposure to upwelling events (5-7 days) as well as testing for possible effects of tidal variability vs. mean variability. For some molluscs (mussels) semidiurnal variability can alleviate low pH_{Ox} effects (Frieder et al. 2014).

The statolith geochemistry of embryonic squid from the laboratory experiments of Chapter 3 was affected by environmental pH_{Ox} (i.e. U:Ca). Because squid statoliths provide a continuous ontogenetic record, this finding supports the possibility that adult squid exposed to low pH_{Ox} can be distinguished from those exposed to ambient pH_{Ox}. If this can be confirmed in the field, it may be possible to conduct a range-wide survey to estimate the relative percentage of the population impacted by low pH_{Ox}. Also, analyses of squid statoliths can be used by the industry and managers to make informed decisions to sustain the fishery by using geochemistry and statolith size to infer the environmental conditions at the natal sites for these squid embryos. For example, if a squid from a heavily fished area are all impacted by low pH_{Ox} at their natal sites, then managers may

opt to lower the fishing quota to minimize possible negative effects associated with fishing mortality and low pHOx sublethal effects. Further, because market squid are a forage species (Morejohn et al. 1978, Lowry and Carretta 1999) this information might also facilitate inshore ecosystem management.

ENSO Paradox

An ENSO paradox emerges from the results of this dissertation. One prediction that could be made from the slowed development observed under low pHOx conditions is that embryonic squid should do poorly during La Niña compared to El Niño. This appears to directly contradict fishery-dependent (Porzio 2013) and fishery-independent data for the population to date. Squid population size appears to be elevated during La Niña and much lower during El Niño (Reiss et al. 2004, Koslow and Allen 2011). This is an intriguing finding and although it is tempting to speculate, currently the data do not exist to resolve this apparent “paradox.”

Rather than to hypothesize about why this is (which is unknown), a more fundamental step should be to standardize methods. Okutani and McGowan (1969) stated that during the 1950s CalCOFI methods to sample squid paralarvae appeared to only modestly reflect *D. opalescens* embryo abundance and may not accurately assess squid paralarval abundance. Since then, CalCOFI methods have been improved by utilizing manta tows at night to sample squid paralarvae (Koslow and Allen 2011). These tows have been taken since 1981 and represent the longest time-series of *D. opalescens* known to exist in the Southern California Bight. It is assumed manta tows are capable of capturing squid paralarvae representative of the squid population. Testing this assumption

will improve the accuracy of the method and standardize for comparison to other methods.

To determine net accuracy, a mesocosm experiment could be conducted within a large seawater aquarium, such as that at Southwest Fisheries Science Center, La Jolla, USA. This testing could quantify the effectiveness of various nets, including those used for manta tows, over a gradient of squid paralarval densities. Then, assessment methods from Okutani and McGowan (1969) could be repeated by comparing embryo abundance at embryo beds proximal to CalCOFI stations to paralarvae abundance determined using manta tows at those stations. These comparisons could not only help to resolve the current paradox but also may enable the CalCOFI time series to be utilized for absolute abundance estimates.

Challenges to Assessing *D. opalescens* Abundance

Currently, the only absolute abundance estimate for *D. opalescens* is derived from the egg-escapement model which is based on fishery-dependent data of spawning adults captured in the fishery harvests (Dorval et al. 2013). Unfortunately, when and where squid are harvested and is primarily driven by economics (e.g. gas prices, high ex-vessel prices paid only for the freshest squid) and squid are only captured when they are profitable for the fishery occurring only when squid aggregate in large numbers. Thus, fishery-dependent data can be susceptible to decoupling from actual abundance. For example, fishers are limited to fishing areas that are in general < 24 hours steam away from port (or < 300 km). Per the California market squid fishery management plan, these ports are located in San Pedro, Port Hueneme, and in Monterey, USA. Thus, using the

catch data biases abundance estimates towards these locations. This means that the Channel Islands such as San Miguel, San Nicolas, Santa Barbara and San Clemente are likely under sampled. Further, fishers will only attempt to harvest when squid aggregate in groups of > 20,000 individual squid (≥ 1 m ton). In stark contrast, successful squid spawning is not dependent on groups totaling 20,000 or more (Chapter 2). There is no evidence to suggest that this economic threshold is important ecologically. Thus fishery-dependent studies completely ignore small squid spawning aggregations, when these aggregations may play an important role in squid population dynamics. Therefore, until the role of squid spawning aggregation size within population dynamics is known, it is difficult to use fishery-dependent data for squid abundance estimates. Currently, there is no way to know whether or not fishery-dependent data is decoupled from squid absolute abundance.

One of the potentially most powerful methods for fishery-independent sampling of *D. opalescens* is through the CalCOFI time series (Koslow and Allen 2011). Using this time series, investigators have clearly identified that the environment impacts *D. opalescens* paralarvae in the Southern California Bight and that the population declines in association with El Niño (Koslow and Allen 2011). Further, the CalCOFI data reveal that the highest densities of paralarvae are found inshore near the Channel Islands during the month of April (Koslow and Allen 2011). There is compelling evidence to suggest that the CalCOFI time series, which is presently underutilized, might offer a more directed assessment of *D. opalescens* abundance, as it does for other key commercial fishes of the SCB.

One of the challenges with the current sampling design of CalCOFI is that of detecting paralarvae at low densities. Okutani and McGowan first identified this problem when contrasting abundance estimates from CalCOFI paralarval samples to benthic embryo abundances (1969) and Koslow and Allen (2011) improved sampling analyses by utilizing the manta nets. Sampling of *D. opalescens* paralarvae at higher spatio-temporal resolution led Zeidberg and Hamner (2002) to a similar conclusion about CalCOFI sampling as Okutani and McGowan (1969). They noted that CalCOFI does not sample much of the available spawning areas because CalCOFI stations are 30 km apart (i.e. under sampled in space); they are also under sampled in time because squid spawn continuously (Chapter 2) and samples are taken only once every three months. Benthic surveys of embryo beds (Chapter 2) can potentially fill in these sampling “holes” and can supplement paralarval sampling if methods can be standardized.

If so, a clear solution to assessing abundance (and resolving the ENSO paradox) is to integrate ROV assessment of embryo beds near CalCOFI stations. Currently benthic surveys of embryo habitat have not been integrated into CalCOFI sampling nor are any plans being made to do this in the future. However, development of this program would standardize methods as well as increase communications between squid regulators and CalCOFI. Standardizing methods would also improve accuracy of squid abundance assessment. If successful, it would also improve certainty and predictability for jobs related to the squid fishery.

Fishing Aggregations and “Egg” Escapement

The commercial extract of a forage species requires thoughtful management because forage species dynamics can impact large portions of the ecosystem (Morejohn et al. 1978). *D. opalescens* is unique among California’s commercially captured forage species in that it is semelparous and that its spawning aggregations are targeted by fishers. These two facts are important for several reasons. First, *D. opalescens* dies shortly after spawning. The industry promotes a low-impact fishery however this assertion is based on the fact that the impact of spawning squid to the ecosystem is unknown (at least energetically). It is known that CA sea lions depend on market squid during their pupping season (Lowry and Carretta 1999) but the degree of dependence of predators on these squid is unknown for most species. Are most species that can eat squid obligate predators or are they facultative predators? Clearly, the energy dynamics of the system needs study before the impact of this fishery can be meaningfully assessed in terms of ecosystem management.

In addition, fishers target squid first arriving at the spawning ground because there is an economic incentive to capture squid with thick mantles that have good “color.” Squid are graded while being offloaded and harvested squid with the thickest mantles and best color receive the highest prices. In contrast, spent squid that have thin mantles and poor color receives low prices. That is, squid that were able to spawn receive the least amount of money compared to those that have spawned the least. Although not quantified, it is unlikely that targeting squid that have yet to spawn or have only started to spawn has a “low impact” on the squid population. Any low impact is likely due to the fishers not being able to capture squid when they first arrive to the spawning grounds

(squid avoidance) not because the fishers are not trying. However, as fishing methods continue to improve, fishers might be able to more successfully target newly arriving squid on the spawning grounds and if so this could rapidly lead to population declines.

Assessment of fishing effects on the squid population are estimated using histological study of “egg escapement” based on Macewitz et al. (2004; the term “egg” often is used in the literature to refer to fertilized eggs or embryos). However, this method cannot be used to evaluate fishing activities in real time and if overfishing is identified, it is done so only after the damage has occurred. At best, this method can identify overfishing activities but does not provide an option for managers to prevent overfishing activities from occurring because the analysis is done retroactively. Further, histological methods are a theoretical escapement method and need validation in the field. It is possible that this method yields a superficially higher proportion of embryo escapement compared to those that actually escape in the field (Ex. Squid often lay embryo filled capsules within nets as they are being captured). Comparing theoretical embryo escapement (based on adult histology) to empirical estimates of embryo escapement is needed to test histological egg escapement methods.

One potential solution can be borrowed from the salmon fishery. The Alaska Department of Fish and Game and the Pacific Fisheries Marine Council count individual salmon at fixed spawning grounds. Using these data, these agencies assess escapement by counting individual salmon at the spawning grounds and then quantify the number of fish that “escape” fishers. The findings of Chapter 2 show that, at least in principle, quantifying individual squid might be a viable solution to test and improve theoretical escapement.

As a first step towards this type of practice, core-embryo habitat needs to be mapped in areas where squid are fished. Mapping should be prioritized in areas where squid are fished most heavily. Once identified, core areas should be protected immediately through spatial-restriction methods. One example could be to make a mandate through the CA market squid fishery management plan that restricts fishing to depths ≥ 40 m depth while allowing for time to development of more refined techniques (e.g. automated underwater vehicle surveys). For example, automated underwater vehicle surveys should be explored as a possible means for rapid assessment.

There is a storied history of fishers targeting spawning aggregations and these fished populations rapidly declining shortly thereafter. This includes population declines associated with groupers (Coleman et al. 1996, Sala et al. 2001), reef fish (Sadovy and Domeier 2005) and temperate bass species (Erisman et al. 2011). Perhaps the most common reason spawning aggregations are overfished is due to hyperstability or the “illusion of plenty” (Sadovy and Domeier 2005). Spawning aggregations are often predictable and catches can increase even with constant catch per unit effort and/or as the population declines (Erisman et al. 2011). As such, spawning aggregations are infamously difficult to correctly quantify and fishery-dependent data can be very difficult to correctly interpret as can fishery-independent data (Sadovy and Domeier 2005). Currently, the accuracy of *D. opalescens* abundance estimates is not resolved enough to determine whether or not the fishery is in decline, stable or increasing (Dorval et al. 2013). Although the spatio-temporal dynamics of *D. opalescens* spawning aggregations are only starting to be understood (Chapter 2), what is known is that when spawning

aggregations are overfished, the affected population can rapidly decline and restoration efforts can be difficult (Sala et al. 2001).

Management Options in California

This dissertation has shown that squid spawn continuously (Chapter 2). These findings highlight the need for squid-embryo habitat to be meaningfully included in research efforts. Currently, management treats the fishery as a single population and the squid population is largely evaluated through landings (Porzio 2013), the CalCOFI time series (Koslow and Allen 2011), and from groundfish surveys as by-catch (Reiss et al. 2004). Inclusion of embryo-habitat research would not only improve the fishery management but also has the potential to assist with ecosystem management. Few studies have assessed the role of squid in the ecosystem but those that have been conducted have provided evidence that squid strongly interact with many species (Morejohn et al. 1978, Lowry and Carretta 1999). Five options are readily available to help managers protect embryo habitat from unintentional fishing mortality and would include the environment in management policies.

(1) Gear restriction. Shortening the height of the seine nets to 20 m or less in the Southern California Bight would eliminate accidental scrapping of squid embryo capsules, as most of the embryo beds are at depths deeper than 20 m (Chapter 2). Protection is needed throughout embryogenesis, which in La Jolla typically takes ~4 weeks in the shallows (≤ 40 m depth) and about 5 weeks at greater depths (> 40 m depth; based on pH_{OX} levels, Chapter 3). Lower temperature increases the development duration as well; development might take as long as 8 weeks at 10 °C or as short as 3

weeks at 16 °C (Zeidberg et al. 2011a). Longer embryogenesis increases the probability that embryo capsules will be dislodged. If this occurs at any point during embryogenesis, this would likely result in death for all encapsulated embryos. Management-mandated gear restrictions could be highly effective at preventing seine nets (or any other nets) from disturbing squid embryo beds. This restriction is in alignment with the fishery management plan (Henry et al. 2005) and would help to prevent accidents where nets unintentionally scrape the seafloor. Even a “light” lead line is sufficient to dislodge all the embryo capsules it contacts. Protecting these embryos means protecting the harvest to come ~6 months later.

(2) Map and monitor squid embryo beds: Embryo bed habitat is much more restricted in area than the habitat available for adults or other life stages. Low [O₂] is a characteristic of eastern boundary currents relative to other boundary current types. One untested hypothesis is that *D. opalescens* prefers the SCB for its relatively high [O₂] relative to other regions of the CCS. Presumably these squid migrate during their last stage of life and select sites for spawning that optimize the survival and fitness of their offspring. Although it would greatly benefit fishery and ecosystem managers, fishery-independent mapping of the squid embryo beds has not been conducted over the range of *D. opalescens*.

As such, how *D. opalescens* utilizes the regions and subregions of the CCS and the relative importance of the squid within SCB relative to the total population is unknown. Despite not understanding these population dynamics (and associated consequences), squid are primarily harvested within the Bight (Porzio 2013). Further, it is unknown how important spawning aggregations and hatchlings are to upper-shelf

ecosystems (energy dynamics). It is known that sea lions are negatively affected when they do not have access to squid (Lowry and Carretta 1999). However, there are many other organisms that depend on squid (Morejohn et al. 1978). How do these interactions change throughout the year? The study of energy dynamics related to squid spawning events would likely provide key insights into the CCS ecosystem dynamics.

Monitoring the mapped embryo beds would also allow scientists to test hypotheses about climate change effects on *D. opalescens*. Monitoring in each section of the CCS would provide insights as to how current structure, expanding hypoxia, and increasingly corrosive waters affect squid population dynamics. Further, each region has excellent research facilities and have documented the occurrence of squid embryo beds (but not the frequency of occurrence; Barkley Sound Canada: Shimek et al. 1984; Monterey Bay, USA: Fields 1965, Ziedberg 2011; La Jolla, USA: McGowan 1954, Okutani and McGowan 1969, Zeidberg 2011, Chapter 2). The environmental exposures experience by the embryos remains largely undescribed outside of La Jolla, USA (Chapter 2). However, managers may only need to organize and to communicate information to the scientists at the many research institution that dot the coastline through each region of the CCS from Canada, through the USA and to Mexico. Depending on findings, small or large areas may need to be protected when harsh conditions affect the populations. Currently, without this information across broad spatial and temporal scales, managers cannot make informed decisions about the impacts of climate change on *D. opalescens*.

(3) Utilize the CalCOFI time series. CalCOFI data can be “calibrated” to derive absolute abundance of *D. opalescens*. With absolute abundance, the CalCOFI

environmental data set will allow evaluation of environmental change impacts on the population on an annual basis every year.

(4) Analyze *D. opalescens* statoliths. Squid statoliths are randomly subsampled from commercially caught fish by California Department of Fish and Wildlife (Porzio pers. comm.). Geochemical analysis of these samples may provide information regarding the sublethal exposure to environmental [O₂] and pH (Chapter 4 - Navarro et al. 2014) as well as information regarding migration trajectories throughout their range (Warner et al. 2009). Statolith ring counts can also help to determine the hatch dates that occur throughout the year (Jackson and Domeier 2003). The frequency and magnitude of *D. opalescens* spawning throughout the year may be important to their persistence.

Statolith analysis is a relatively inexpensive means to analyze captured squid. It can provide information about squid hatch date and how squid age relates to the catch (Jackson and Domeier 2003). These robust techniques can be applied to any squid sampled not just harvested squid (Chapter 2). Managers can also use this information to justify conducting higher-resolution studies using other tools available including remotely operated vehicles (ROVs), side-sonar, and/or mark-recapture study for further investigation.

(5) New research initiatives should be conducted in collaboration with fishers when possible. Current efforts should be encouraged so that scientists and fishers both benefit. Closures represent opportunities for research that are currently underutilized. In South Africa, managers close the squid fishery (*Loligo vulgaris reynaudii*) every summer to conduct physical and biological oceanography field work (Roberts personal communication). A lottery allows a portion of the squid industry to work for pay to help

researchers. A similar effort could be implemented in California. Weekend closures could be used to survey distribution of embryo capsules useful for assessing whether or not embryos are experiencing their optimal temperature and oxygen levels. In addition, they can be used to estimate the number of adults that spawned in the area (total squid spawning aggregation). This is particularly useful to evaluate the number of squid captured as a percentage of the total squid spawning aggregation. This method would provide managers with rare insight into the impacts of fishing on a spawning ground.

When the fishery reaches its quota, surveys can be conducted to evaluate embryo habitat. This may help understand the impact of light boats on adult site selection. Chapter 2 reports that recreational divers observed squid migrated towards light boats and away from their dive sites. If light boats change squid site selection, then by how much? Thus, light boat fishers could help advance understanding of the influence of light boats on spawning squid site selection or squid behavior in MPAs. Gathering information to address these questions is of mutual benefit to fishers and scientists. When fisher-scientist collaboration occurs, a productive culture develops that allows scientists to advance understanding of the fishery (and research publications) while fishers have a sustainable harvest as well as steady income and employment.

Given that the *D. opalescens* resource presently provides the state of California's largest fishery (by volume; Porzio 2013), a focused research and management program, that incorporates new gear restrictions, mapping embryo-bed habitat, climate change, long-term monitoring, and greater interaction with the fishers is well justified.

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APPENDIX 1.

Comparison of Loliginid Fisheries Among Boundary Current Ecosystems

In this appendix I will compare market squid (*D. opalescens*) spawning aggregation site selection and embryo habitat to those of two other loliginid species. I will discuss possible environmental impact for each of the three squid fisheries: the California, USA market squid fishery (*Doryteuthis opalescens*), the Argentina squid fishery (*Doryteuthis gahi*), and the Republic of South Africa chokka squid fishery (*Loligo vulgaris reynaudii*). These fisheries were chosen because they represent the largest loliginid fishery in three current boundary types (i.e. western, eastern and transition boundary currents). Eastern boundary currents are represented by the California Current, USA, western boundary currents by Brazilian Current, Argentina, and the transition zone between the Agulhas and Benguela Currents along the east cape of the Republic of South Africa (Figure 1). In California, market squid fishery policy acknowledges the importance of the environment on the *D. opalescens* fishery and mandates inclusion into regulations (see market squid fishery management plan, Henry et al. 2005).

The objectives of this appendix are to consider the environmental influence on the California squid fishery and how it relates to: (1) environmental influence on other loliginid fisheries, (2) squid spawning aggregations and the encapsulated life stages and (3) management options and practices utilized internationally.

I will consider such questions as: How do the life histories of the different fishery species differ and how are they similar? Does this affect their vulnerability to climate

change? Globally, are loliginid fishery managers moving towards the inclusion of environmental factors and climate change into fisheries management and, if so, what tools, conceptual issues, data, and time scales are being included?

California, USA Market Squid Fishery (*Doryteuthis opalescens*)

The oceanographic conditions vary across the California Current System (CCS) according to relative strength of the currents within each region (Checkley and Barth 2009). Thus the climate change impacts on squid may differ regionally. This species of squid has an inshore distribution throughout the northern, central and southern CCS. They occur from British Columbia, Canada across the coast of all the western states of the U.S.A. and extending from Baja California to Baja California Sur, Mexico. Sometimes these squid can occur north of British Columbia into south-eastern Alaska (Vojkovich 1998).

Chapter 2 reported that the O₂, pH, T and salinity varied widely within the La Jolla embryo bed across seasons and years. This is consistent with many studies that show that the environment of the Southern California Bight is dynamic (Frieder et al. 2012, Nam et al. 2011, Send and Nam 2012). Spawning adults selected sites consistent with the hypothesis that they choose habitat with high O₂ and pH (relative to habitat utilized by non-spawning adults) and are impacted by biotic interactions at the upper portions of their habitat. In British Columbia, Canada (Barkley Sound Region), *D. opalescens* regularly selects shallow sites during the summer (Shimek et al. 1984) and can be collected intertidally (Vince Levesque pers. comm.). In Monterey, USA *D. opalescens* embryos are found at an intermediary depth (22 m depth; Zeidberg et al.

2011). It is an open question as to whether the same predation pressures and/or competition for space exist in Canada or even Monterey, USA as in the Southern California Bight, however it is known that the seawater with analogous O₂ and pH levels shoal to shallow depths in the summer (Crawford and Peña 2013). Fishing records are consistent with O₂ –induced compression of squid aggregations as the catch is often the highest during years with low O₂ in surface waters (Porzio 2013, Vojkovich 1998). Further, theory suggests that low O₂ can lead to squid migration (Pauly 2010) driven by physiology (Portner 2010) and if so, *D. opalescens* habitat would be constrained throughout the Southern California Bight (Zeidberg 2011).

In Chapter 2, squid were shown to be associated with the 25.8 σ_t isopleth (water density). Studies show that in southern California, O₂ levels associated with 25.6 σ_t are declining but it is unclear how much is due to climate change or due to other anthropogenic activities (Booth et al. 2014). If *D. opalescens* embryo habitat requires high O₂, then spawning squid may respond to O₂ declines by spawning at increasingly shallower depths in southern California, as occurs in the northern CCS (Barkley Sound Region, Canada).

Republic of South Africa Chokka Squid Fishery (*Loligo vulgaris reynaudii*)

In the Republic of South Africa, the chokka squid fishery, *Loligo vulgaris reynaudii*, is relatively new and commercial exploitation started in the 1980s (Sauer 1983). Chokka are bigger and can reach sizes of up to 400 mm in dorsal mantle length compared to *D. opalescens* (130 mm DML is a large market squid; Chapter 2). *L. vulgaris reynaudii* spawn over a longer duration (months) compared to *D. opalescens*,

whose individuals spawn over days to weeks. The habitat utilized by chokka squid is limited by visibility (Sauer 1992), temperature and O₂ levels, but not substrate type (Roberts 2005). They occur in the transition zone along the south-eastern cape where oxygen levels are high (Roberts et al. 2005). They can occur on coarse sandy substrate and low relief (Augustyn 1990), usually from 20-110 m depth but also at depths to 270 m (Roberts et al. 2012). When bottom temperatures are ~12 °C at mid-shelf regions (119 m depth), *L. vulgaris reynaudii* embryos survived in similar rates as those that develop on the upper shelf (20 m depth; Oosthuizen and Roberts 2009). *L. vulgaris reynaudii* embryo habitat is limited by optimal [O₂] (150-210 μM) and temperature (12-17 °C, Roberts 2005). *L. vulgaris reynaudii* currently spawns where this optimal [O₂] and temperature coincide. If warming were to intensify, the spawning habitat could become degraded.

Argentina inshore commercial squid fishery (*Doryteuthis gahi*)

The first descriptions of *D. gahi* embryo beds were published in 2001 (Baron 2001). Although *D. gahi* and *D. sanpaulensis* co-occur along the coast in northern Argentina, only *D. gahi* is fished commercially; *D. sanpaulensis* is caught as by catch in the artisanal fishery. Both *D. gahi* and *D. sanpaulensis* embryos occur at depths of 15 m or less. *D. gahi* attaches embryo capsules to hard substrate whereas *D. sanpaulensis* embryo capsules were only found on sandy substrate. *D. gahi* occurs at Nuevo Gulf, Argentina every month. The bottom temperature is seasonal at Nuevo Gulf, ranging from 12 to 18 °C (Ortiz et al 2011). These temperatures are driven by seasonal cycles associate with the Brazilian Current (Ortiz et al. 2011). *D. gahi* embryos have also been

observed at depths of 65-70 m (Laptikhovsky 2007) and off of the Falkland Islands, southern populations of *D. gahi* embryo habitat experiences temperatures between 4-10 °C from 20-100 m depths (Arkhipkin et al. 2004). In the laboratory setting, optimal temperature for *D. gahi* embryos to develop into paralarva is 5-20 °C (Baron 2002). This is the largest optimal temperature range of any of the squid species discussed and may be a unique characteristic to *D. gahi*.

Fishers and Scientists

Management of the three fisheries discussed in this chapter differs. For example, South Africa actively formed its commercial squid fishery to create jobs whereas the USA and Argentina developed management to sustain ongoing fisheries. This difference is pronounced enough to alter the relationship between researchers and fishers and change the 'culture' of the fishery. In South Africa, fishers and researchers continue to work together, even during fishery closures. Every year the fishery is closed for 5-6 weeks to allow spawning aggregations to lay their embryo capsules. During this season a lottery is opened to the fishers and two fishing vessels and crew are selected for hire to assist research efforts. These fishers help to capture squid for researchers for tracking studies (Navarro pers. observ.). Fishers and researchers may not always like one another, but they seem to recognize that more benefits occur from working together than not.

The reason is that fishers and the researchers benefit from open communication channels that would not exist without their research collaborations. For example, fishers openly go to spawning sites that researchers might not otherwise know about. In

exchange, researchers provide new scientific information about those sites that fishers might not otherwise ever know. South African scientists clearly benefit as they have produced +60 research articles and are leaders in the study of squid fisheries biology. Fishers benefit by having job security that occurs with a sustainable fishery.

In California, USA the fishers generally avoid researchers. This is changing with new research initiatives conducted through the California Wetfish Producers, Inc. (D. Porzio pers. comm). Along with the potential to contribute to science, the industry effort value in these types of collaboration lies with the bridge of communications between fishers and scientists. This is unusual in west coast fisheries, as previous collaborations have been driven by necessity and through rebuilding and restoring overfished fisheries. For example, in 2000 rockfish on the west coast were overexploited, largely due to trawl fisheries, to the point of being declared a disaster by the Secretary of Commerce. Since 2006 and the reauthorization of the Magnuson-Stevens Act, groundfish have been monitored through a collaboration between fishers and NOAA scientists working as collaborators in U.S. West Coast Groundfish Bottom Trawl Survey (www.nwfsc.noaa.gov). New collaborations occurring between scientists and the squid fishery offer promise that fishers and scientists can work together to ensure practices that sustain the squid population from being overfished *and to sustain jobs*.

In Argentina, the management of the squid fishery is just beginning to become species-specific. Prior to 2012, fishery receipts would be generally designated as “squid” making assessment of *Doryteuthis gahi* difficult to quantify because there are three co-occurring species *D. gahi*, *D. sanpaulensis*, and *Illex argentinus*. (Ortiz and Crespi pers. comm.). However, these scientists are changing Argentine policies and now species-

specific data for catch amount, date of catch, and location is being recorded in Argentina. This is important to global fisheries because not only does Argentina have a large and active *D. gahi* fishery but also it is host to the largest squid fishery in the world over the last decade, the *Illex argentinus* fishery (Crespi and Baron 2012). In addition, at the northern latitudes of Argentina, another loliginid co-occurs, *D. sanpaulensis* (Baron 2001, 2003a, 2003b). *D. sanpaulensis* is often caught as by-catch by artisanal fishers (Baron 2003a). Argentina is moving towards more study of *D. gahi* and other squid fishery species (Crespi, personal communication).

Global Trends

Market squid are just one of many examples of inshore squid. Globally, inshore squid are important players in marine ecosystems. Of all the teuthioids (squid), loliginids (such as *D. opalescens*) are perhaps the most uniquely adapted for the inshore environment. They usually reside over or near the shelf, and are completely dependent on the shelf seafloor as habitat for their offspring (Boyle and Rodhouse 2005). A characteristic common to 40 species within Loliginidae (Anderson 2000) is the formation of spawning aggregations on the shelf. Loliginids are commonly forage species in their respective ecosystems and are a viable source of protein for many taxa. Globally, species from this family are commonly harvested for commercial use.

Loliginid fisheries are emerging at one of the fastest rates within the booming global squid fishery (Jereb and Roper 2010). Loliginids are often highly desirable for human consumption and make up around 19% (1.6 million tons) of the total global cephalopod harvest of 8.3 million tons (Jereb and Roper 2010). In areas where coastal

pelagic fish such as sardine and anchovy have been depleted, loliginids are thought to increase in biomass (Fields 1973, Caddy and Rodhouse 1998). Although it is clear loliginids need protection from overfishing, it is less clear how these species will be impacted by climate change.

The loliginid species examined exhibit surprisingly similar optimal temperature and oxygen concentrations for their embryo beds. Another commonality is that these embryo bed habitats are fixed and small relative to the area occupied by other life stages. For loliginids inhabiting eastern boundary currents, $[O_2]$ (and to a lesser extent pH/pCO_2) may constrain the area and location of essential embryo habitat (Chapter 2). In Chapter 2, *D. opalescens* realized embryo habitat was limited by that $[O_2]$ levels to the upper shelf intermittently over the shelf whereas *D. opalescens* was not limited by temperature (which is optimal at all depths of the shelf year round). Further, it is reported in Chapter 2 that spawning *D. opalescens* target $[O_2]$ -rich areas on the shelf (rather than spawning on the slope). Along the north-eastern Pacific, the Southern California Bight represents an area with the high $[O_2]$ concentrations available to *D. opalescens*. The relative importance of *D. opalescens* SCB subpopulations to the overall *D. opalescens* population remains a key question for future research and for the sustainability of the market squid fishery.

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Figure

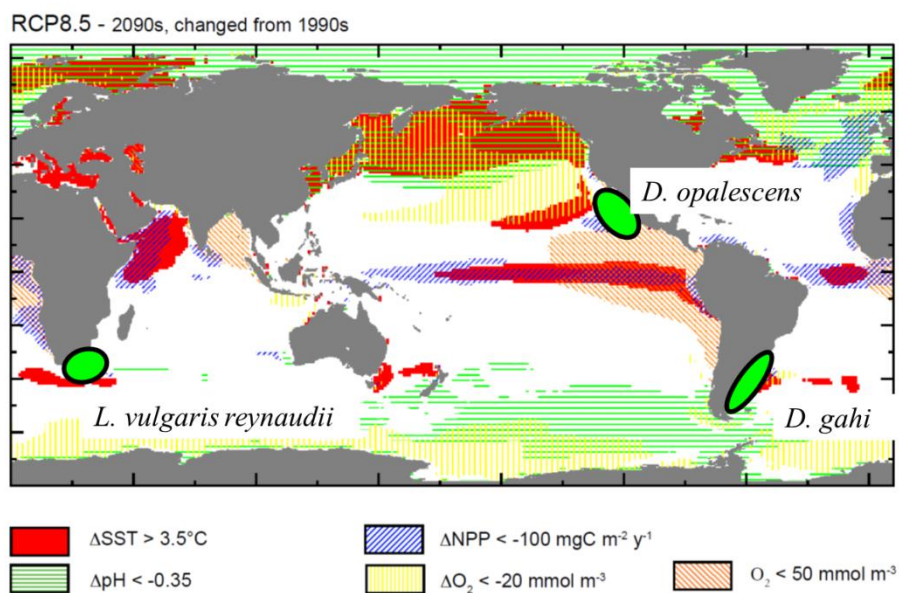


Figure A.1. Adapted from Bopp et al. 2013. Global map depicting predicted changes for climate across the world ocean to occur by 2090-2099. Bright green circles indicate loliginid fisheries discussed in this chapter. *D. opalescens* = California market squid fishery. *D. gahi* = Argentina inshore squid fishery. *L. vulgaris reynaudii* = South African chokka squid fishery.