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The Effects of Ocean Acidification and Upwelling Conditions on the Growth and Calcification of the Red Abalone (_Haliotis rufescens_)

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Chapter 1 – Thesis Introduction

Background

Since the industrial revolution, the earth’s atmospheric CO$_2$ has increased from 280 ppm to about 390 ppm and is still rising at an increasing rate of about 0.5% per year (Orr et al., 2005). An increase of 100 ppm is within the naturally oscillating range of atmospheric CO$_2$; however, a variation of that magnitude has previously only occurred between 180 ppm and 280 ppm, and on a much larger time scale, between ice ages and interglacials (Tyrrell, 2008). In fact, atmospheric CO$_2$ levels are higher today than Earth has experienced in at least the last 800,000 years (Lüthi et al., 2008), and are still rising at a rate approximately 100 times faster than has occurred in the last 650,000 years (Royal Society, 2005).

This rapid increase in atmospheric CO$_2$ has already caused significant chemical changes in the marine environment. The ocean acts as a natural sink for CO$_2$, having taken up about 25% of anthropogenic CO$_2$ emissions since the industrial revolution (Sabine et al., 2004). While this slows the increase of atmospheric CO$_2$, the impacts to the ocean could be devastating. CO$_2$ dissolves from the atmosphere into the ocean due to the difference between partial pressures of CO$_2$. This causes a drop in ocean pH, which is known as ocean acidification. Since the industrial revolution, average surface ocean pH has decreased by 0.1 units (Feely et al., 2004). Under the “business-as-usual” emissions scenario, it is projected that the ocean’s average surface pH will drop another 0.3-0.4 units by 2100, and eventually be reduced by 0.7 units, resulting in larger pH changes in the ocean than have been experienced in the last 300 million years, (Caldeira and Wickett, 2003; Orr et al., 2005).

When CO$_2$ increases in the atmosphere, the ocean absorbs increasing amounts at the air-sea interface, where it enters the inorganic carbon system, consisting of three main reactions:
Aqueous carbon dioxide, carbonic acid (H₂CO₃), bicarbonate (HCO₃⁻), and carbonate ion (CO₃²⁻), comprise what is known as the total dissolved inorganic carbon, or DIC. These reactions are known as the inorganic carbon system that largely regulates the pH of seawater. Seawater is generally comprised of 0.5% CO₂, 13% carbonate ion, and 86.5% bicarbonate. As CO₂ concentration increases in seawater, carbonate ion concentrations and pH decrease (Zeebe and Wolf-Gladrow, 2001). In addition, carbonate ion will directly react with CO₂ in the following reaction:

\[
\text{CO}_2 + \text{CO}_3^{2-} + \text{H}_2\text{O} \rightleftharpoons 2 \text{HCO}_3^{-}
\]

This process leads to a further decrease in carbonate ion and increase in bicarbonate, lowering the pH of seawater further (Orr et al., 2005).

In addition to DIC, total alkalinity (TA) is another measurement that is used to quantify inorganic carbon seawater chemistry. TA is defined as the number of moles of hydrogen ion equivalent to the excess of proton acceptors over proton donors in one kilogram of seawater (Dickson, 1981). TA includes the charged molecules in the carbonate system (H⁺, HCO₃⁻, and CO₃²⁻), as well as B(OH)₄⁻, OH⁻, and other charged minor components. Measurements of DIC and TA can be used to calculate the \(p\text{CO}_2\) and pH in seawater (Zeebe and Wolf-Gladrow, 2001).

**Calcium Carbonate Formation and Dissolution**

As a result of climate change and increased ocean acidification, marine organisms are currently being exposed to a rapidly changing environment. Calcifying invertebrates form calcium carbonate (CaCO₃) to build shells and other skeletal structures necessary for ecological roles in their life cycles, such as protection from predators and structural stability (Fabry et al., 2008). Calcium carbonate is formed in the following net reaction:
\[
\text{Ca}^{2+} + 2 \text{HCO}_3^- \rightleftharpoons \text{CaCO}_3 + \text{CO}_2 + \text{H}_2\text{O}
\]

This net formation requires two bicarbonate ions and one calcium ion, reducing TA and DIC at a 2:1 ratio (Zeebe and Wolf-Gladrow, 2001). Since CO$_2$ is produced by this reaction, calcification actually increases the concentration of CO$_2$ in seawater, though it is rapidly converted to bicarbonate. The reverse reaction is also true, which means that an abundance of CO$_2$ enhances the dissolution rate of calcium carbonate.

Calcite and aragonite are the two most common forms of CaCO$_3$ found in marine organisms. Considering only the physical chemistry, whether aragonite and calcite form or dissolve in seawater depends on the saturation state of each in seawater, which is a function of calcium ion and carbonate ion concentrations. The aragonite saturation state of seawater, $\Omega_{\text{arag}}$, is defined by the following equation:

$$
\Omega_{\text{arag}} = \frac{[\text{Ca}^{2+}]_{\text{sw}} \times [\text{CO}_3^{2-}]_{\text{sw}}}{K_{sp(\text{arag})}^{*}}
$$

where $K_{sp(\text{arag})}^{*} = [\text{Ca}^{2+}]_{\text{sat}} \times [\text{CO}_3^{2-}]_{\text{sat}}$ in seawater that is saturated with respect to aragonite.

When $\Omega_{\text{arag}} > 1$, seawater is supersaturated with respect to aragonite and calcification is favored. When $\Omega_{\text{arag}} < 1$, seawater is undersaturated and dissolution is favored; however, some organisms can calcify even when $\Omega_{\text{arag}} < 1$ by redirecting more energy into the formation of calcium carbonate than it would otherwise require in water supersaturated with respect to aragonite; however, this may come at a cost to other physiological functions.

Since calcium ion concentrations in the ocean generally vary only slightly (with salinity), the saturation state is mostly dependent on the carbonate ion concentration (Zeebe and Wolf-Gladrow, 2001). The same rules apply for calcite; however, less carbonate ion is required to saturate seawater with respect to calcite than aragonite due to a difference in the crystal structure. This means that $K_{sp}^{*}$ is lower for calcite than for aragonite; therefore, seawater becomes
undersaturated with respect to aragonite before it is undersaturated with respect to calcite. This causes aragonite to be approximately 50% more soluble than calcite in seawater (Mucci, 1983).

The depth at which aragonite or calcite begin to dissolve is called the saturation horizon. The saturation horizon of aragonite is shallower than that of calcite. As carbonate ion concentrations decrease in the upper ocean due to CO₂ uptake, the aragonite and calcite saturation horizon becomes shallower. Carbonate ion concentrations are projected to decrease by 60% in high latitudes by the end of the century, and the aragonite saturation state is projected to reach 0.72, a decrease of 0.68, above 50° N in the Pacific Ocean (Orr et al., 2005). In the past 200 years, the saturation horizon of aragonite has already shoaled by 40 to 100 meters in the North Pacific and by 30 to 200 meters in the North Atlantic (Feely et al., 2004). It has been measured to be only 100 to 300 meters from the surface at its shallowest point in the northeastern Pacific Ocean during a May cruise, allowing waters undersaturated with respect to aragonite to be transported onto the continental shelf during upwelling events (Feely et al., 2008); however, these findings need to be confirmed with long-term monitoring near the coast of the Pacific throughout to year to establish the locations, magnitude, and frequencies of upwelling events.

Upwelling occurs all along the western coast of North America and is a result of winds moving surface waters offshore, which forces deep waters onto the continental shelf (Feely et al., 2008). Upwelling generally begins in early spring and lasts until late summer or fall. A natural increase in pCO₂ in deep waters results from upwelling because increased nutrients in upwelled waters lead to plankton blooms in the shallows, which sink to the bottom and decompose. The deep waters that well up during this period are cold, high in dissolved CO₂, and low in dissolved oxygen (DO). This presents three environmental stressors that affect organisms that inhabit the
subtidal and intertidal along the Pacific coast. Water at depths less than 50 meters becomes undersaturated in aragonite during upwelling events in certain parts of the California coast (Feely et al., 2008). During 1995 and 1997 spring upwelling events, coastal areas near Santa Cruz, California were observed to experience surface $p$CO$_2$ levels near 700 µatm (Friederich et al., 2002). This may be due to the shoaling of the aragonite saturation horizon from the addition of anthropogenic CO$_2$ emissions to the already high $p$CO$_2$ in deep waters. In a 5-year study, Nam et al. (in press), observed that the aragonite saturation state reached about 50 m during summer upwelling events off Del Mar, California and falls well below saturation at 35 m for two months during La Niña events.

With rising atmospheric CO$_2$ and the ocean’s increased anthropogenic CO$_2$ uptake, the aragonite saturation horizon may become shallower and waters undersaturated with respect to aragonite could be forced to the surface in larger areas, with greater frequency, and for longer durations during upwelling events. If this occurs, it could severely impact the calcifying capabilities of shallow species or larval stages of benthic species that do not have the evolutionary adaptations to live in acidified waters. More research is needed on the magnitudes, durations, and frequencies of upwelling and the depths of the aragonite saturation horizon in coastal areas before the potential threats to coastal species can be evaluated.

**Potential Effects of Ocean Acidification on Calcifying Organisms**

Ocean acidification is occurring so rapidly that it may be impossible for many populations and species to adapt to the changes. Impacts to calcifying species in particular may be significant if ocean pH and carbonate ion concentrations continue to decrease as projected (Fabry et al., 2008). Many benthic invertebrates begin their lives in the plankton and require the formation of a calcium carbonate shell in order to settle to the seafloor and metamorphose into
the next stages of their lives. Survival into adulthood and reproductive maturity may be hindered without suitable shells for development and protection from predators.

Several studies on the effects of ocean acidification on calcifying organisms have focused on either tropical or polar regions of the ocean. Polar and subpolar regions are projected to become undersaturated in aragonite by the year 2050 due to cold water’s ability to absorb carbon dioxide more quickly than warmer water, and polar species are projected to experience high levels of shell dissolution (Orr et al., 2005). Tropical regions are often studied due to the threat to reef-building corals and their calcium carbonate structures, which are essential to the high biodiversity in low latitudes (Turley et al., 2007). However, relatively few studies have been done on the effects of ocean acidification with relation to upwelling on temperate coastal species. Temperate areas are also likely to be affected by ocean acidification in the near future, particularly in areas that experience upwelling events, such as the coast of California. Conditions along the California coast are highly variable throughout the year, and coastal organisms are well adapted to fluctuations in temperature, pH, and oxygen levels within a certain range; however, this tolerance range may not be wide enough to allow all species to survive in waters undersaturated with respect to aragonite. The marine ecosystems along the California coast provide large economic resources, including fish and shellfish aquaculture and several commercial fisheries, as well as tourism and recreational resources. A great deal more research is needed for species that inhabit coastal temperate areas predisposed to upwelling to evaluate how each species will react to rapid changes in $pCO_2$ when combined with low DO and low temperatures.

Studies suggest that species do not all experience the same effects to acidified conditions; however, the effects found are often limited by the response measured, which varies dramatically
between studies (Lindinger et al., 1984; Miles et al., 2007; McDonald et al., 2009). Most studies on the effect of ocean acidification conditions on mollusks, crustaceans, and echinoderms have demonstrated a negative effect to either physiology, survival, or developmental dynamics (Dupont et al., 2010). Sea urchins exposed to a pH < 7.5 experienced 100% mortality due to inadequate ability to regulate acid-base balance in the coelomic fluid (Miles et al., 2007). Lindinger et al. (1984) found that mussels not only experienced shell dissolution, but also increased hemolymph pH in high pCO₂. Kurihara (2008) observed negative impacts on the fertilization, cleavage, settlement, and reproductive stages in the early development of echinoderms, mollusks, and crustaceans when exposed to increased pCO₂. These studies show that decreased calcification is not the only potential effect ocean acidification can have on benthic invertebrates.

Some studies found no effect or a positive effect from acidified conditions; however, these findings are usually coupled with a negative effect to the organism in some way. McDonald et al. (2009), exposed barnacles to decreased pH and observed an actual increase in calcification of the shell; however, less force was required to penetrate shells of barnacles grown in pH 7.4 than shells of barnacles grown in pH 8.2, indicating that shells were weaker under acidified conditions. Wood et al. (2008) reported that burrowing brittlestars were able to regenerate lost arms in decreased pH, which requires an increase in calcification and metabolism, but muscle mass and function was also negatively affected as a result. The impact of ocean acidification varies depending on the biological process examined. Some calcifying species may possess the ability to withstand decreased pH by redirecting more energy into calcification, thus maintaining their shells in acidified conditions; however, this may only be a short term solution that is not sustainable for the organism over prolonged exposure times. In general, the
biodiversity of benthic invertebrates is predicted to decrease significantly due to the physiological stresses caused by ocean acidification (Widdicombe and Spicer, 2008; Fabry et al., 2008; Dupont et al., 2010).

**Effects of Upwelling Conditions on Abalone**

In-depth research in the field of ocean acidification is imperative to predict future conditions of marine ecosystems under increased CO$_2$, and areas at risk of becoming undersaturated with respect to aragonite in the near future, such as the Pacific coast of the U.S., should be the priority. One California native species that has received little research in the field of ocean acidification is the red abalone (*Haliotis rufescens*). The red abalone is historically a plentiful subtidal species ranging from Sunset Bay, Oregon to Bahia Tortugas, Baja California, Mexico. In the past 30 years, however, overharvesting, parasitism, and the withering syndrome disease have devastated the populations throughout southern and central California (Viant et al., 2003). Intense commercial and recreational fishing depleted red abalone populations between 1969 and 1982, forcing resource agencies to closely manage the fishery in northern California and to close the fishery completely throughout southern California. Management strategies enforcing size and bag limits on abalone have done little to increase the populations throughout California. Since abalone are broadcast spawners whose reproductive success varies from year to year, assuming that they will be reproductively successful for many years before they grow to the size allowed for take is not always valid, especially in populations already suffering from low densities. Years of red abalone recruitment have been found to be as rare as every four years in protected populations (Karpov et al., 1998). In a species that has had limited recovery success since its collapse, the management strategies of the past are not effective enough to recover the species. Because the species is an important fishery and aquaculture market for the state of
California, a better understanding of the impacts ocean acidification and upwelling will have on the red abalone is needed to allow for more thorough preparation for what the species is facing now and in the near future.

Abalone larvae settle out of the plankton in response to environmental stimuli, such as the phycobilins of encrusting coralline algae, red abalone’s primary food source, or conspecific mucus (Slattery, 1992; Gruenthal et al., 2007). Settlement occurs after the formation of the peristomial shell and first respiratory pore at about 6 weeks in age, allowing larvae to sink to the substratum (Leighton, 1974). This step in development triggers metamorphosis into the post-larval or juvenile stage when the shell is usually 1.5 mm-2.5 mm in length (Leighton, 1989). Abalone shells consist of an outer organic layer, called the periostracum, a prismatic layer of calcite and a thin inner layer of nacre, which is about 20 nm thick (Verma et al., 2006). The nacreous layer, also known as mother of pearl, is a matrix of aragonite platelets that supports several types of proteins responsible for continued shell growth and strengthening (Michenfelder et al., 2003). This portion of the shell is particularly vulnerable to a pH decrease in seawater due to aragonite’s thermodynamic instability (Collino and Evans, 2007). If the aragonite layer is compromised by deterioration or dissolution, shell growth and strength will be negatively affected and the abalone may not be as well protected from predators and environmental threats, such as was found by McDonald et al. (2009) in barnacle shells.

The red abalone’s habitat depth ranges from the intertidal to as deep at 30 meters, the broadest depth range of any Pacific abalone species, which overlaps with the depths that experience upwelling. This means that red abalone are exposed to upwelling conditions such as high $pCO_2$ and low DO. Red abalone are thought to spawn year-round (Boolootian et al., 1962), indicating that their gametes, larvae, and early developmental stages would also be exposed to
upwelling conditions during a portion of the year. Previous studies on the red abalone’s response to decreased DO indicate a detrimental effect on growth (Tjeerdema et al., 1991). Harris et al. (1999) found that the juvenile greenlip abalone experienced a 5% and 50% reduction in shell growth in DO saturation levels of 96% (7.32 mg/l), and 77% (5.93 mg/l), respectively, and no shell growth at all with DO levels below 56% saturation (4.32 mg/l). Additional findings suggest that juvenile abalone survival is minimal with DO levels below 42% saturation (3.22 mg/l), after 48 hours of exposure due to an increase in hemolymph pH from increased respiration and hyperventilation (Cheng et al., 2004). DO levels in deep water transported to shallow depths during upwelling events are typically 1.8-3.6 mg/l and have been found to reach hypoxic levels (1.27-1.67 mg/l) (Grantham et al., 2004). During a La Niña upwelling event, Nam et al. (in press), observed DO levels below 4.0 mg/L concurrent with an aragonite saturation state well below one in waters at 35 m off Del Mar, California.

Temperature research on multiple abalone species has indicated that growth rates are more favorable in warmer thermal conditions. Leighton (1974) reported that red abalone eggs develop normally within a temperature range of 10-23°C, but growth of planktonic larvae after hatching is restricted to between 13.5°C and 20°C. The same study found that the post-settlement stage of red abalone survive over a range of 10-19.5°C, but only grow to advanced post-larval stages in 14-18°C. Steinersson and Imsland (2003) repeated that red abalone growth rates are optimal in 14-18°C by measuring shell growth over a range of 11-22°C. Vilchis et al. (2005), on the other hand, observed that growth of the red abalone is most favorable in somewhat colder waters (12-15°C) based on the total mass index and reproductive health. Feely et al. (2008) recorded temperatures as low as 6°C in waters that reached the red abalone’s depth range during upwelling events off Point St. George, California. This could impact the survival of larval red
Abalone, which have been found to have decreased thermal tolerance when exposed to decreased pH (Zippay and Hoffman, 2010). Extremely low temperatures could potentially reduce the growth and survival of young abalone when coupled with increased acidity and low DO levels; however, no studies have yet been published evaluating these interactive effects.

During the juvenile stage, the shell is very thin and, therefore, vulnerable to dissolution. Since the spawning seasons for most abalone species, including the red abalone, coincide with the timing of most upwelling events, early life stages are exposed to upwelling conditions and the effects should be considered. To explore the effects upwelling could have on the red abalone, all stages of the life history should be exposed to factors associated with upwelling, including high $pCO_2$ and low DO. This will give a better indication of what the future may hold for this species and allow conservation agencies to prepare for future risks on a species level.

Although no studies have yet been published that measure the effect of ocean acidification on abalone calcification or dissolution, Harris et al. (1999), demonstrated that the greenlip abalone and the blacklip abalone had decreased growth as a result of decreased pH. Studies conducted on several mollusk species, including pteropods (Orr et al., 2005; Fabry et al., 2008), bivalves (Lindinger et al.; 1984; Green et al., 2004; Michaelidis et al., 2005; Gazeau et al., 2007; Kurihara et al., 2007), snails (Bibby et al., 2007), and cephalopods (Pörtner and Reipschläger, 1996), found decreased growth or fitness as a result of increased $pCO_2$. Calcification rates have been found to decrease in some species even when surface water remains supersaturated with calcium carbonate (Kleypas et al., 1999). Ocean acidification, when combined with overfishing, parasites, disease, and the harsh conditions of upwelling, will expose the red abalone to multiple stressors that could threaten the conservation of this important species.
in its natural habitat. The collapse of the red abalone would, in turn, have profound effects on the California ecosystem and the large aquaculture market.

Since upwelling exposes subtidal benthic invertebrates to elevated $p\text{CO}_2$ levels and low DO levels simultaneously, both of these stressors should be studied to evaluate the cumulative effect on calcifying species. The physiological stress caused by decreased DO may interfere with the ability of benthic invertebrates to compensate for shell dissolution. The energy it requires to cope with both variables at once may affect the ability of benthic invertebrates to compete for food, avoid predation, and survive in nature. As a result of the shoaling aragonite saturation horizon, marine organisms will be exposed to a rapidly changing environment. More research is needed to evaluate the combined effects of upwelling and ocean acidification on calcifying species, as well as to determine the locations, frequencies, and durations of upwelling events along the Pacific coast of the U.S. This information would allow natural resource agencies to adapt their management strategies for specific species that are found to be sensitive to upwelling conditions. The aquaculture industry would also benefit from knowing the sensitivity and responses shellfish species have to upwelling conditions and ocean acidification. A greater understanding of the conditions benthic invertebrates will face and how these conditions may affect them will allow for a more effective management strategy and a better chance for the survival of the species.
**Literature Cited**


Chapter 2

Upwelling and Ocean Acidification: Effects of High CO$_2$ and Low DO on the Growth and Calcification of the Red Abalone (*Haliotis rufescens*)

Abstract

Upwelling events along the California coast expose invertebrates to low dissolved oxygen simultaneously with high $p$CO$_2$ levels that are progressively increasing as a result of rising atmospheric CO$_2$. These multiple stressors could potentially impact the growth and calcification of economically valuable molluscs, such as abalone. To evaluate this threat, juvenile red abalone were maintained over a 4-week period in seawater undersaturated with respect to aragonite and containing 85% dissolved oxygen, which simulated an upwelling event. Seawater conditions were then returned to ambient levels for 3 weeks to determine the ability of the abalone to recover from the potential effects of low oxygen and high $p$CO$_2$ conditions. Abalone exposed to the treatment had lower shell weights and calcium content per shell than abalone in the ambient group. Shells also appeared much lighter in color following the acidification period. After both groups were returned to ambient conditions, shells of the abalone in the treatment group still weighed less and had lower calcium content than the shells of the ambient group. The amount of weight gained by the abalone during the 3-week ambient period, however, was the same for both groups, suggesting an ability to recover a normal rate of weight gain after exposure. These findings suggest that juvenile red abalone experienced decreased net calcification following exposure to high CO$_2$ and decreased DO. Though abalone were able to recover to normal growth rates, they were not able to accelerate their net calcification to catch up to the shells weights and calcium content of the ambient group, suggesting that they may have thinner or less dense shells following each upwelling event.
Introduction

Atmospheric CO₂ has increased by over 100 ppm in the past 200 years, an increase of about 40%, due to the combustion of fossil fuels, deforestation, and land development (Royal Society, 2005). The ocean is a natural sink for atmospheric CO₂ and has absorbed approximately 25% of all anthropogenic CO₂, resulting in a reduction in ocean surface pH by 0.1 units (Sabine et al., 2004). Under the “business-as-usual” emissions scenario, it is projected that the ocean’s average surface pH will drop another 0.3-0.4 units by 2100, and eventually be reduced by 0.7 units, resulting in larger pH changes in the ocean than have been experienced in the last 300 million years, (Caldeira and Wickett, 2003; Orr et al., 2005).

In addition to causing a pH reduction, increasing aqueous CO₂ levels in the ocean result in a decrease of carbonate ion (CO₃²⁻) concentrations, which affects the formation of calcium carbonate (Orr et al., 2005). Calcite and aragonite are the two most common forms of calcium carbonate found in marine organisms. Considering only the physical chemistry, whether aragonite and calcite form or dissolve in seawater depends on the saturation state of each in seawater, which is a function of calcium ion and carbonate ion concentrations (Zeebe and Wolf-Gladrow, 2001). The aragonite saturation state of seawater, Ω_{arag}, is defined by the following equation:

\[
\Omega_{arag} = \frac{[Ca^{2+}]_{sw} \times [CO_{3}^{2-}]_{sw}}{K_{sp(arag)}}
\]

where \(K_{sp(arag)} = [Ca^{2+}]_{sat} \times [CO_{3}^{2-}]_{sat} \) in seawater that is saturated with respect to aragonite.

When \(\Omega_{arag} > 1\), seawater is supersaturated with respect to aragonite and calcification is favored. When \(\Omega_{arag} < 1\), seawater is undersaturated and dissolution is favored; however, some organisms can calcify even when \(\Omega_{arag} < 1\) by redirecting more energy into the formation of calcium carbonate than it would otherwise require in water supersaturated with respect to aragonite.
Since calcium ion concentrations in the ocean generally vary only slightly (with salinity),
the saturation state is mostly dependent on the carbonate ion concentration (Zeebe and Wolf-
Gladrow, 2001). The same rules apply for calcite; however, less carbonate ion is required to
saturate seawater with respect to calcite than aragonite due to a difference in the crystal structure.
This means that seawater with decreasing carbonate ion becomes undersaturated with respect to
aragonite before it is undersaturated with respect to calcite. This causes aragonite to be
approximately 50% more soluble than calcite in seawater (Mucci, 1983).

The depth at which aragonite or calcite begin to dissolve is called the saturation horizon.
The saturation horizon of aragonite is shallower than that of calcite. As carbonate ion
concentrations decrease in the upper ocean due to CO₂ uptake, the aragonite and calcite
saturation horizon becomes shallower. Carbonate ion concentrations are projected to decrease by
60% in high latitudes by the end of the century, and the aragonite saturation state is projected to
reach 0.72, a decrease of 0.68, above 50° N in the Pacific Ocean (Orr et al., 2005). In the past
200 years, the saturation horizon of aragonite has already shoaled by 40 to 100 meters in the
North Pacific and by 30 to 200 meters in the North Atlantic (Feely et al., 2004). It has been
measured to be only 100 to 300 meters from the surface at its shallowest point in the northeastern
Pacific Ocean during a May cruise, allowing waters undersaturated with respect to aragonite to
be transported onto the continental shelf during upwelling events (Feely et al., 2008); however,
these findings need to be confirmed with long-term monitoring near the coast of the Pacific
throughout to year to establish the locations, magnitude, and frequencies of upwelling events.

Upwelling occurs all along the western coast of North America and is a result of winds
moving surface waters offshore, which forces deep waters onto the continental shelf (Feely et al.,
2008). Upwelling generally begins in early spring and lasts until late summer or fall. A natural
increase in $p\text{CO}_2$ in deep waters results from upwelling because increased nutrients in upwelled waters lead to plankton blooms in the shallows, which sink to the bottom and decompose. The deep waters that well up during this period are high in dissolved $\text{CO}_2$ and low in dissolved oxygen (DO). This presents two environmental stressors that affect organisms inhabiting the subtidal and intertidal along the Pacific coast. Water at depths less than 50 meters becomes undersaturated in aragonite during upwelling events in certain parts of the California coast (Feely et al., 2008). During 1995 and 1997 spring upwelling events, coastal areas near Santa Cruz, California were observed to experience surface $p\text{CO}_2$ levels near 700 $\mu \text{atm}$ (Friederich et al., 2002). This may be due to the shoaling of the aragonite saturation horizon from the addition of anthropogenic $\text{CO}_2$ emissions to the already high $p\text{CO}_2$ in deep waters. In a 5-year study, Nam et al., (in press), observed that the aragonite saturation state reached about 50 m during summer upwelling events off Del Mar, California and falls well below saturation at 35 m for two months during La Niña events.

With rising atmospheric $\text{CO}_2$ and the ocean’s increased anthropogenic $\text{CO}_2$ uptake, the aragonite saturation horizon may become shallower and waters undersaturated with respect to aragonite could be forced to the surface in larger areas, with greater frequency, and for longer durations during upwelling events. If this occurs, it could severely impact the calcifying capabilities of shallow species or larval stages of benthic species that do not have the evolutionary adaptations to live in acidified waters. More research is needed on the magnitudes, durations, and frequencies of upwelling and the depths of the aragonite saturation horizon in coastal areas before the potential threats to coastal species can be evaluated.

Temperate areas are likely to be affected by ocean acidification in the near future, particularly in areas that experience upwelling events, such as the coast of California (Feely et
al., 2008). Conditions along the California coast are highly variable throughout the year, and coastal organisms are well adapted to fluctuations in temperature, pH, and oxygen levels within a certain range; however, this tolerance range may not be wide enough to allow all species to survive in waters undersaturated with respect to aragonite. The marine ecosystems along the California coast provide large economic resources, including fish and shellfish aquaculture and several commercial fisheries, as well as tourism and recreational resources.

Studies suggest that species do not all experience the same effects to acidified conditions; however, the effects found are often limited by the response measured, which varies dramatically between studies (Lindinger et al., 1984; Miles et al., 2007; McDonald et al., 2009). Most studies on the effect of ocean acidification conditions on mollusks, crustaceans, and echinoderms have demonstrated a negative effect to either physiology, survival, or developmental dynamics (Dupont et al., 2010). These studies show that decreased calcification is not the only potential effect ocean acidification can have on benthic invertebrates. However, some studies found no effect or a positive effect from acidified conditions. McDonald et al. (2009), exposed barnacles to decreased pH and observed an actual increase in calcification of the shell; however, less force was required to penetrate shells of barnacles grown in pH 7.4 than shells of barnacles grown in pH 8.2, indicating that shells were weaker under acidified conditions. Wood et al. (2008) reported that burrowing brittlestars were able to regenerate lost arms in decreased pH, which requires an increase in calcification and metabolism, but muscle mass and function was also negatively affected as a result. Some calcifying species may possess the ability to withstand decreased pH by redirecting more energy into calcification, thus maintaining their shells in acidified conditions; however, this may only be a short term solution that is not sustainable for the organism over prolonged exposure times. In general, the biodiversity of benthic invertebrates
is predicted to decrease significantly due to the physiological stresses caused by ocean acidification (Widdicombe and Spicer, 2008; Fabry et al., 2008; Dupont et al., 2010).

In depth research in the field of ocean acidification is imperative to predict future conditions of marine ecosystems, and areas at risk of becoming undersaturated with respect to aragonite in the near future, such as the Pacific coast of the U.S., should be the priority. One California native species that has received little research in the field of ocean acidification is the red abalone \((Haliotis rufescens)\). The red abalone is a historically plentiful subtidal species ranging from Sunset Bay, Oregon to Bahia Tortugas, Baja California, Mexico. In the past 30 years, however, overharvesting, parasitism, and the withering syndrome disease have devastated the populations throughout southern and central California (Viant et al., 2003). Intense commercial and recreational fishing depleted red abalone populations between 1969 and 1982, forcing resource agencies to closely manage the fishery in northern California and to close the fishery completely throughout southern California. Management strategies enforcing size and bag limits on abalone have done little to increase the populations throughout California. Since abalone are broadcast spawners whose reproductive success varies from year to year, assuming that they will be reproductively successful for many years before they grow to the size allowed for take is not always valid, especially in populations already suffering from low densities. Years of red abalone recruitment have been found to be as rare as every four years in protected populations (Karpov et al., 1998). In a species that has had limited recovery success since its collapse, the management strategies of the past are not effective enough to recover the species. Because the species is an important fishery and aquaculture market for the state of California, a better understanding of the impacts ocean acidification and upwelling will have on the red
abalone is needed to allow for more thorough preparation for what the species is facing now and in the near future.

Abalone shells consist of an outer organic layer, called the periostracum, a prismatic layer of calcite and a thin inner layer of nacre, which is about 20 nm thick (Verma et al., 2006). The nacreous layer, also known as mother of pearl, is a matrix of aragonite platelets that supports several types of proteins responsible for continued shell growth and strengthening (Michenfelder et al., 2003). This portion of the shell is particularly vulnerable to a pH decrease in seawater due to aragonite’s thermodynamic instability (Collino and Evans, 2007). If the aragonite layer is compromised by deterioration or dissolution, shell growth and strength may be negatively affected and the abalone may not be as well protected from predators and environmental threats.

The red abalone’s habitat depth ranges from the intertidal to as deep as 30 meters, the broadest depth range of any Pacific abalone species, which overlaps with the depths that experience upwelling, meaning that red abalone are exposed to upwelling conditions such as high pCO₂ and low DO. Red abalone are thought to spawn year-round (Boolootian et al., 1962), indicating that their gametes, larvae, and early developmental stages would also be exposed to upwelling conditions during a portion of the year. Previous studies on the red abalone’s response to decreased DO indicate a detrimental effect on growth (Tjeerdema et al., 1991). Harris et al. (1999) found that the juvenile greenlip abalone experienced a 5% and 50% reduction in shell growth in DO saturation levels of 96% (7.32 mg/l), and 77% (5.93 mg/l), respectively, and no shell growth at all with DO levels below 56% saturation (4.32 mg/l). DO levels in deep water transported to shallow depths during upwelling events are typically 1.8-3.6 mg/l and have been found to reach hypoxic levels (1.27-1.67 mg/l) (Grantham et al., 2004). During a La Niña
upwelling event, Nam et al. (in press), observed DO levels below 4.0 mg/L concurrent with an aragonite saturation state well below one in waters at 35 m off Del Mar, California.

Temperature research on multiple abalone species has indicated that growth rates are more favorable in warmer thermal conditions. Leighton (1974) reported that red abalone eggs develop normally within a temperature range of 10-23°C, but growth of planktonic larvae after hatching is restricted to between 13.5°C and 20°C. The same study found that the post-settlement stage of red abalone survive over a range of 10-19.5°C, but only grow to advanced post-larval stages in 14-18°C. Steinersson and Imsland (2003) repeated that red abalone growth rates are optimal in 14-18°C by measuring shell growth over a range of 11-22°C. Vilchis et al. (2005), on the other hand, observed that growth of the red abalone is most favorable in somewhat colder waters (12-15°C) based on the total mass index and reproductive health. Feely et al. (2008) recorded temperatures as low as 6°C in waters that reached the red abalone’s depth range during upwelling events off Point St. George, California. This could impact the survival of larval red abalone, which have been found to have decreased thermal tolerance when exposed to decreased pH (Zippay and Hoffman, 2010). Extremely low temperatures could potentially reduce growth and survival of young abalone when coupled with increased acidity and low DO levels; however, no studies have yet been published evaluating these interactive effects.

During the juvenile stage, the shell is very thin and, therefore, vulnerable to dissolution. Since the spawning seasons for most abalone species, including the red abalone, coincides with the timing of most upwelling events, early life stages are exposed to upwelling conditions and the effects should be considered. To explore the effects upwelling could have on the red abalone, all stages of the life history should be exposed to factors associated with upwelling, including high $pCO_2$, low dissolved oxygen (DO), and low temperatures. This will give a better indication
of what the future may hold for this species and allow conservation agencies to prepare future risks on a species level.

Although no studies have yet been published that measure the effect of ocean acidification on abalone calcification, Harris et al. (1999) demonstrated greenlip and blacklip abalone had decreased growth as a result of decreased pH. Studies conducted on several other mollusk species, including pteropods (Fabry et al., 2008; and Orr et al., 2005), bivalves (Lindinger et al.; 1984; Green et al., 2004; Michaelidis et al., 2005; Gazeau et al., 2007; Kurihara et al., 2007), snails (Bibby et al., 2007), and cephalopods (Pörtner and Reipschläger, 1996), found decreased growth or fitness as a result of increased \( p\text{CO}_2 \). Calcification rates have been found to decrease in some species even when surface water remains supersaturated with calcium carbonate (Kleypas et al., 1999). Ocean acidification, when combined with overfishing, parasites, disease, and the harsh conditions of upwelling, will expose the red abalone to multiple stressors that could threaten the conservation of this economically important species in its natural habitat.

Since upwelling exposes subtidal benthic invertebrates to elevated \( p\text{CO}_2 \) levels and low DO levels simultaneously, both of these stressors should be studied to evaluate the cumulative effect on calcifying species. The physiological stress caused by decreased DO may interfere with the ability of benthic invertebrates to compensate for shell dissolution. The energy it requires to cope with both variables at once may affect the ability of benthic invertebrates to compete for food, avoid predation, and survive in nature. As a result of the shoaling aragonite saturation horizon, marine organisms will be exposed to a rapidly changing environment. More research is needed to evaluate the combined effects of upwelling and ocean acidification on calcifying species, as well as to determine the locations, frequencies, and durations of upwelling events.
along the Pacific coast of the U.S. This information would allow natural resource agencies to adapt their management strategies for specific species that are found to be sensitive to upwelling conditions. The aquaculture industry would also benefit from knowing the sensitivity and responses shellfish species have to upwelling conditions and ocean acidification. A greater understanding of the conditions benthic invertebrates will face and how these conditions may affect them will allow for a more effective management strategy and a better chance for the survival of the species.

**Methods**

**Dissolved Gas Manipulation System**

In order to expose abalone to high $p$CO$_2$ and low DO levels in a controlled manner, an automated aquarium system was built to manipulate and maintain the dissolved gas levels in seawater. Six Omega mass flow controllers were used to control the amount of O$_2$, CO$_2$, and N$_2$ gas added to each gas mixture (Figure 1; Omega FMA 5418 (0-5 SLM) for N$_2$, Omega FMA 5502 (0-10 SCCM) for CO$_2$, and Omega FMA 5411 (2 SLM) for O$_2$). Three of these mass flow controllers sent a gas mixture to the control aquaria and the other 3 sent a separate gas mixture to the treatment aquaria. They were controlled using a custom built program developed with National Instruments’ Labview software and Data Acquisition Devices (DAQs). This allowed for dissolved gas levels to be controlled by entering the desired percentage of each of the 3 gases. The mass flow controllers sent each gas mixture to the corresponding aquaria through Liquicels®, which are plastic cartridges that ran seawater from each aquarium across membranes that equilibrated it with the gas mixture without producing bubbles in the aquaria. Each aquarium’s seawater was recirculated constantly through a Liquicel® to maintain the desired
dissolved gas level throughout the experiment. Temperatures were controlled using two Lauda waterbaths, which pumped chilled water through submerged titanium coils in each tank. This method was used to maintain each tank’s temperature within the red abalone’s optimal growth range between 12 and 18˚C observed by Leighton (1974), Steinersson and Imsland (2003), and Vilchis et al. (2005).

Figure 1. Mass Flow Controllers used to control both the treatment gas levels and the ambient gas levels within each aquarium.

Two 30 gallon aquaria were designed and built, each with an impermeable acrylic wall in the center, resulting in four 15 gallon aquaria. In addition, each aquarium contained a compartment with a mesh screen to separate the abalone from the pumps, titanium coils, and drain lines. The seawater used was pumped from an ocean intake off Scripps Pier, through a settling tank, and into a holding tank at Birch Aquarium. Each aquarium had its own 10 μm filter stage, which was cleaned and replaced as necessary, before water was pumped through the Liquicels (Figure 2). Fresh seawater drained into each aquarium at a controlled rate of 0.8
gallons per hour. This allowed for complete turnover of seawater about every 19 hours in each aquarium.

Figure 2. Set up of 4 aquariums with Liquicels® positioned above positioned above. Ambient aquaria on right, treatment aquaria on left. Seawater was pumped from aquaria through a 10 µm filter, and into the Liquicel® to equilibrate with the set gas mixture before returning to the aquaria.

Location and Conditions

The study was carried out at the UCSD Scripps Institution of Oceanography’s Stephen Birch Aquarium in La Jolla, California. About 450 juvenile abalone were obtained from the Cultured Abalone aquafarm in Goleta, California for the experiment. The abalone were 7-13 mm in length and 5-8 mm in width upon arrival at Birch. They were acclimated in two flow-through 15 gallon aquaria for two days prior to initiation of the experiment. This period of time was assumed to be adequate based on the minimal to non-existent death rate on the second full day of acclimation.

Following acclimation, abalone were removed from the aquaria at random to be measured, weighed, and tagged. Marking tags were applied to the apex of the shell of each
abalone using cyanoacrylate glue. The lengths and widths of the abalone shells were measured to the nearest 0.01 mm using Fisher Scientific digital calipers (0-150 mm, accuracy ±0.03 mm, precision ±0.01 mm). Wet weights were obtained (after tagging), by drying individual abalone of excess seawater and weighing in aluminum weigh boats on a Metro Toledo XS205 Dual Range Analytical Balance (accuracy ±0.01 mg). These measurements were recorded as the initial conditions of each abalone according to the color and number on the tag. Abalone were then placed in the aquarium based on their tag color: Ambient 1 (A1), Ambient 2 (A2), Treatment 1 (T1), and Treatment 2 (T2), so that each aquarium had 105 abalone (Figure 3).

After tagging and measuring all the abalone, 5 abalone were removed at random from each aquarium for an initial subset. These 20 abalone were placed in a freezer for about an hour to aid in complete tissue removal, then the shells and tissue were separated, weighed, and dried at 55°C overnight. Dried abalone were removed from the oven into a dessicator and dry weights of the shells and tissues were obtained. Shells were saved for calcium analysis.
Acidification Period

The 4-week acidification period of the study consisted of setting the dissolved gas levels in the T1 and T2 to 85% DO and around 1200 µatm pCO₂, or high enough to maintain an undersaturation of aragonite. A1 and A2 were set to around 400 µatm pCO₂ and 100% DO; however, these values were all adjusted based on the daily monitoring feedback received during the experiment. Each aquarium was cleaned daily and received exactly 1 teaspoon of fish food (Tetramin flakes). Dead abalone were removed immediately. Daily seawater samples were bottled and sealed with 100 µL mercuric chloride. Seawater samples were analyzed every 5 days for dissolved inorganic carbon (DIC; accuracy ±0.1 µmol/kg; precision ±0.01 µmol/kg) using a UIC, Inc. coulometer, and for total alkalinity (TA; accuracy ±0.02 µmol/kg; precision ±0.01 µmol/kg) using an automated titration system. Certified reference material samples were used as standards to calibrate for both DIC and TA determination (Dickson et al., 2003). Salinities were also run for each seawater sample. Seawater parameters (DIC and TA) were then entered into CO2System to obtain the pCO₂, pH_{total}, Ω_{arag}, and Ω_{cal} in each sample. Standard deviation of DIC and TA were calculated using the daily measured values in each parameter to obtain the day-to-day variation in DIC and TA within each aquarium. The standard deviations for pCO₂, pH, Ω_{arag}, and Ω_{cal} were obtained using the calculated values for each daily seawater sample. Aquaria were also monitored daily with a Durafet pH/temp probe (total scale), and gas and waterbath settings were adjusted accordingly to maintain consistent conditions. DO was monitored daily using a Hach LDO probe (accuracy ±0.05 mg/L; precision ±0.01 mg/L).

Abalone exposed to the treatment were observed to appear more lethargic than the abalone in the ambient aquaria. Abalone were observed to begin moving as soon as they settled on a surface following a disturbance. To quantify this behavior, ten abalone were individually
removed every week from each aquarium at random and placed on a plexiglass grid with 1 cm squares. Abalone were allowed to settle for a few seconds and then the number of squares that they crawled were counted over one minute time periods before they were returned to their aquaria. These scores were then averaged for each aquarium and time period. Each abalone’s distance crawled was used to obtain a mean “activeness score.” These scores were compared between treatment groups to evaluate the effect of the high $pCO_2$ and decreased DO treatment on fitness (Figure 4).

Figure 4. Abalone being observed for its activeness score over a one minute time interval on a grid of 1 cm squares.

On the last day of the acidification period, 40 abalone were removed from each aquarium, weighed for their final wet weight, and immediately frozen. After about an hour, tissues were removed from the shells and each abalone was dried overnight at 55°C. Shell measurements, tissue dry weights, and shell dry weights were recorded according to each abalone’s tag. Shells were preserved for calcium analysis.
Recovery Period

To initiate the recovery period, the settings for T1 and T2 were altered from 1200 \( \mu \text{atm} \) \( p\text{CO}_2 \) and 85% DO to ambient levels (400 \( \mu \text{atm} \) \( p\text{CO}_2 \) and 100% DO). Dissolved gas levels were allowed to adjust gradually by recirculating the existing seawater through the Liquicel\textsuperscript{®} with the new gas mixture. The settings for A1 and A2 remained at ambient levels. The first day of the recovery period, all remaining abalone were removed from the aquaria and measured for length, width, and wet weight, then returned to their respective aquaria for 3 weeks. These measurements were subtracted from the initial measurements to obtain the growth of each abalone during the acidification period. Seawater samples were taken daily and analyzed in the same manner as during the acidification period.

After 3 weeks, ten abalone were pulled from each aquarium for the activity scores in the same manner as during the acidification portion. All abalone were then removed from the aquaria and measured for wet weight, length, and width. The wet weights, lengths and widths recorded at the beginning of the recovery period were then subtracted from the final measurements to obtain the growth of each abalone during the recovery period. They were then frozen for an hour before tissue and shells were separated and dried at 55\(^\circ\)C overnight. Tissue and shell dry weights were obtained and 20 abalone from each aquarium were prepared and saved for element analysis. The remaining shells were preserved for Calcium analysis.

Calcium Analysis

Shells that were separated from tissue after each time period were analyzed for Calcium content with a PerkinElmer 3700 Optical Emission Plasma Spectrometer (+0.001 ppb). Samples were prepared by dissolving pre-weighed individual shells with a known weight of 70% nitric acid in glass scintillation vials. Shells were allowed to dissolve overnight, and the resulting fluid
was diluted twice to a 1:100 ratio. Samples were analyzed for calcium and the resulting number was multiplied by the dilution factors to maintain the original calcium content of each abalone shell.

**Statistical Analysis**

Results were analyzed using the software program R. Separate linear models were run for each time period using dry weight of the whole abalone, dry tissue weight, shell weight, wet weight gained, length grown, width grown, and calcium content per shell as response variables after meeting assumptions of normality. The aquarium each abalone was in was used as a nested variable within treatment to account for any variability between the aquaria of each treatment group. Shell length was included as a nuisance parameter in all linear models since it was highly significant in all analyses (p<0.01).

**Results**

**Acidification Period**

During the 4 week acidification period, seawater in the treatment aquaria maintained average aragonite saturation states of less than one (Ω_{arag}=0.92 and Ω_{arag}=0.93), and pCO₂ means of about 1142 µatm and 1120 µatm in T1 and T2, respectively (Table 1). The calcite saturation states averaged Ω_{cal}=1.43 (+0.20), in the T1 and Ω_{cal}= 1.45 (+0.21), in T2. The ambient aquaria maintained a mean aragonite saturation state above one (Ω_{arag}=1.83 and Ω_{arag}=1.87), and mean pCO₂ of about 484 µatm and 468 µatm in A1 and A2, respectively. In A1, Ω_{cal}=2.87 (+0.29), and Ω_{cal}=2.92 (+0.24) in A2. The mean temperatures (+SD), in each aquarium were 13.6°C (+0.9, T1), 13.5°C (+0.9, T2), 13.5°C (+0.6, A1), and 13.3°C (+0.6, A2).
Table 1. Means of daily seawater samples from each aquarium during the 4-week acidification period (N=28 per aquarium). Standard deviation (in parentheses), is between daily seawater samples that were measured for DIC, TA, and DO. Other parameters shown were calculated using the DIC and TA for each daily seawater sample with standard deviation for the variation between calculated values for each sample. DIC measurements: accuracy $\pm 0.1$ µmol/kg; precision $\pm 0.01$ µmol/kg. TA measurements: accuracy $\pm 0.02$ µmol/kg; precision $\pm 0.01$ µmol/kg.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DIC (µmol/kg)</th>
<th>TA (µmol/kg)</th>
<th>$p$CO$_2$ (µatm)</th>
<th>pH$_{total}$</th>
<th>$\Omega_{arag}$</th>
<th>$\Omega_{cal}$</th>
<th>DO(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>2156(±17)</td>
<td>2201(±10)</td>
<td>1142(±180)</td>
<td>7.62(±0.07)</td>
<td>0.92(±0.13)</td>
<td>1.43(±0.20)</td>
<td>84.0(±1.7)</td>
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<tr>
<td>T2</td>
<td>2154(±15)</td>
<td>2202(±11)</td>
<td>1120(±194)</td>
<td>7.63(±0.07)</td>
<td>0.93(±0.13)</td>
<td>1.45(±0.21)</td>
<td>83.7(±1.8)</td>
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<tr>
<td>A1</td>
<td>2046(±13)</td>
<td>2206(±12)</td>
<td>484(±63)</td>
<td>7.96(±0.05)</td>
<td>1.83(±0.19)</td>
<td>2.87(±.29)</td>
<td>93.6(±1.5)</td>
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<tr>
<td>A2</td>
<td>2041(±13)</td>
<td>2205(±11)</td>
<td>468(±46)</td>
<td>7.97(±0.04)</td>
<td>1.87(±0.15)</td>
<td>2.92(±0.24)</td>
<td>94.8(±1.8)</td>
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</tbody>
</table>

Growth in shell length (linear model, $p=0.6140$; $F=0.2548$; $Df_1=1$; $Df_2=364$), and width (linear model; $p=0.9966$; $F=0.0001$; $Df_1=1$; $Df_2=364$), did not differ significantly between treatment groups (data not shown); however, the treatment group gained an average of 8.5 mg (9%), less in wet weight (shell and tissue), than the ambient group after 4 weeks of exposure after correcting for differences in length and variation between aquaria within each treatment (Figure 5; $p=0.02537$; $F=5.0400$; $Df_1=1$; $Df_2=364$).
Figure 5. The square root amount of wet weight gained during the acidification period was significantly lower for the abalone receiving high CO₂ and low DO than the abalone receiving ambient levels when compared against the shell length (linear model; \( p=0.02537; \, F=5.0400; \, Df_1=1; \, Df_2=364 \)). Treatment abalone gained an average of 9% less over the acidification period than the ambient group when correcting for length and aquarium variability.

The dry weights were also significantly less for the treatment group than the ambient group by an average of 7.4 mg (6%), \( (p=1.472\times10^{-7}; \, F=30.364; \, Df_1=1; \, Df_2=154) \). This difference in dry weight can most likely be attributed to the difference in shell weight, which was 5.9 mg (7%), lower on average in the treatment group than the ambient group (Figure 6; \( p=1.372\times10^{-10}; \, F=47.413; \, Df_1=1; \, Df_2=154 \)), unlike the tissue dry weights, which were only 1.5 mg (4%) lighter on average in the treatment abalone when accounting for length and aquarium variability \( (p=0.5307; \, F=0.3949; \, Df_1=1; \, Df_2=154) \). This indicates that the abalone in the treatment aquaria
maintained the same rate of shell growth as the abalone in the ambient aquaria, but the shells were less dense or thinner in the treatment aquaria.

Figure 6. The Log_{10} dry shell weights following the acidification period were significantly lower for the abalone exposed to high CO₂ and low DO than for the abalone exposed ambient levels when accounting for differences in length (linear model; p=1.372x10^{-10}; F=47.413; Df₁=1; Df₂=154). Treatment shells weighed an average of 7% less than the ambient shells when correcting for length and aquarium variability.

This is confirmed by the calcium content per shell, which was lower by 2.4 ppt (9%), in the treatment shells than the ambient shells (Figure 7; p=2.305x10^{-5}; F=23.813; Df₁=1; Df₂=35). There was significant variability between the ambient aquaria for this analysis. The shells of the abalone in the treatment aquaria were visibly lighter in color, some appearing almost white, when compared to the ambient aquaria’s shells (Figure 8). After one week of the acidification
period, the treatment group’s mean activity score of 16.40 (SD±4.09) cm/min was significantly higher than the ambient group’s mean score of 10.15 (SD±3.34) cm/min (p=5.426x10^{-6}; F=27.936; Df_{1}=1; Df_{2}=38). This may be interpreted as a shock response from the treatment abalone for their first week of exposure to treatment conditions; however, following the first week, the treatment group scores dropped to be about the same as the ambient group scores and followed the same trend thereafter (Figure 12; p=0.5852; F=0.3031; Df_{1}=1; Df_{2}=38).

Figure 7. The square-root calcium content per shell (ppt), was significantly lower in the shells of the abalone exposed to high CO_{2} and low DO than in the shells of the abalone exposed to ambient levels when compared against lengths of the shells (linear model; p=2.305x10^{-5}; F=23.813; Df_{1}=1; Df_{2}=35). Treatment shells contained an average of 9% less calcium than ambient shells when correcting for length and aquarium variability.
Figure 8. Comparison of abalone shell color from each aquarium following the acidification period. Top row exposed to ambient conditions. Bottom row exposed to high CO₂ and low DO. Treatment shells prismatic layer appeared much lighter in color than the ambient group after 4 weeks of exposure indicating dissolution at the surface of the shell.

**Recovery Period**

During the 3 week recovery period, all aquaria were returned to ambient seawater chemistry conditions (Table 2). All aragonite saturation states were kept above one and DO was returned to near 100% saturation. Mean temperatures (+SD) in each aquarium were 14.0°C (+0.4, T1), 14.1°C (+0.4, T2), 14.0°C (+0.6, A1), and 13.8°C (+0.5, A2).
Table 2. Means of daily seawater samples from each aquarium during the 3-week ambient period (N=22 per aquarium). Standard deviation (in parentheses), is between daily seawater samples that were measured for DIC, TA, and DO. Other parameters shown were calculated using the DIC and TA for each daily seawater sample with standard deviation for the variation between calculated values for each sample. DIC measurements: accuracy ±0.1 µmol/kg; precision ±0.01 µmol/kg. TA measurements: accuracy ±0.02 µmol/kg; precision ±0.01 µmol/kg. T1, T2= Aquaria were previously receiving high CO2 and low DO for 4 weeks before being switched to ambient levels during recovery period. A1, A2= Aquaria received ambient levels the entire experimental period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DIC (µmol/kg)</th>
<th>TA (µmol/kg)</th>
<th>pCO2 (µatm)</th>
<th>pH_{total}</th>
<th>Ω_{arag}</th>
<th>Ω_{cal}</th>
<th>DO(%)</th>
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<tr>
<td>T1</td>
<td>2035(±23)</td>
<td>2216(±6)</td>
<td>437(±64)</td>
<td>8.00(±0.05)</td>
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<td>2041(±20)</td>
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<td>464(±59)</td>
<td>7.98(±0.05)</td>
<td>1.95(±0.19)</td>
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<td>2027(±23)</td>
<td>2219(±11)</td>
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<td>8.03(±0.05)</td>
<td>2.14(±0.22)</td>
<td>3.35(±0.34)</td>
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<tr>
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<td>2214(±4)</td>
<td>421(±59)</td>
<td>8.02(±0.05)</td>
<td>2.08(±0.21)</td>
<td>3.25(±0.33)</td>
<td>95.9(±1.2)</td>
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</tbody>
</table>

Growth in shell length (p=0.9535, F=0.0034; Df₁=1; Df₂=196), and width (p=0.9125; F=0.0121; Df₁=1; Df₂=196), again, did not differ between groups during the recovery period (data not shown). The amount of wet weight gained was still 4.7 mg (10%), less on average for the treatment group than the ambient group during the recovery period; however, there was too much variability within each treatment to be significant (Figure 9; p=0.7886; F=0.0721; Df₁=1; Df₂=186). There was a significant variability between A1 and A2 for this response analysis. This suggests that the majority of the abalone that had been exposed to the treatment in the acidification period were able to recover to about the same rate of weight gain as the ambient
group. Despite having about the same weight gain rate as the ambient groups, the treatment
groups had lower dry weights by an average of 6.6 mg (4%), (p=7.318x10^{-10}; F=41.702; Df_1=1;
Df_2=209), and lower shell weights of 4.2 mg (4%), (Figure 10; p=6.742x10^{-12}; F=52.969; Df_1=1;
Df_2=209), than the ambient groups after the recovery period when accounting for variation in
length and aquarium.

Figure 9. The square root amount of wet weight gained during the recovery period was not
significantly different between treatment groups accounting for length and aquarium variation
(linear model; p=0.7886; F=0.0721; Df_1=1; Df_2=186). Treatment abalone gained an average of
10% less than the ambient abalone during the recovery period when correcting for length and
aquarium variability; however, this relationship was not significant due to the amount of
variability within the treatment groups.
Figure 10. Log$_{10}$ shell dry weights of the shells of the abalone that were exposed to high CO$_2$ and low DO were significantly less than the shells of the abalone in the ambient aquaria even after the recovery period when accounting for length and aquarium variation (Log-transformed linear model; p=6.742x10$^{-12}$; F=52.969; Df$_1$=1; Df$_2$=209). Treatment shells were an average of 4% lighter than ambient shells when correcting for length and aquarium variability.

The calcium content per shell was still lower by 2.3 ppt (6%), in the treatment shells compared to the control shells (Figure 11; p=0.001125; F=12.596; Df$_1$=1; Df$_2$=35). The tissue weights, again, did not differ between groups (p=0.0748; F=3.2061; Df$_1$=1; Df$_2$=209).
Figure 11. Square root calcium content per shell in the abalone initially exposed to high CO₂ and low DO was still lower than the abalone in the ambient aquaria when compared against shell length following recovery period (Square root-transformed linear model; \( p=0.001125; F=12.596; \) \( Df_1=1; Df_2=35 \)). Treatment shells calcium content averaged 6% less than ambient shells when correcting for length and aquarium variability.

The difference between activity scores for the end of the recovery period and the end of the acidification period were not significantly different between treatment groups (\( p=0.2610; F=1.3067; Df_1=1; Df_2=34 \)). The activity scores of both groups were higher following the recovery period, most likely as a result of their increased size, with a mean of 17.55 cm/min in the ambient group and 21.11 cm/min in the treatment group (Figure 12; \( p=0.0852; F=3.1323; Df_1=1; Df_2=36 \)).
Figure 12. Time series of abalone mean activity scores over experimental period (N=40 per time period). Time points are one week, 2.5 weeks, and 4 weeks after the initiation of the acidification period, and at the end of the recovery period (week 7). Error bars are ±SE.

Shells of the treatment groups appeared less washed out than they were following the acidification period, and were closer to the color of the ambient aquaria’s shells (Figure 13). This may indicate a recovery of some of the shell that may have been lost over the acidification period.
Figure 13. Comparison of abalone shells from each aquarium following both the acidification and the recovery period. Left side shells from ambient aquaria; right side from treatment aquaria. Top two rows were removed from the aquaria following the acidification portion; bottom two rows were removed following the recovery period. Treatment shells appeared lighter in color than the ambient group following exposure to high CO$_2$ and low DO, but gained some color back after the recovery period.

**Discussion**

This is the first study of its kind to evaluate the additive effects of high CO$_2$ and low DO on a benthic species. In addition, while most ocean acidification studies generally take infrequent seawater measurements and look only at the precision of the DIC, TA, and/or pH measurements to calculate the variability in the seawater, this study used high precision measurements and daily seawater samples to estimate the day-to-day variability that the animals experienced with great accuracy. After exposure to high $p$CO$_2$ and decreased DO, juvenile red abalone showed a decreased net calcification in comparison with the ambient group. This was confirmed by lower
shell weights as well as lower calcium content per shell than found in the shells of abalone exposed to ambient conditions. Although the treatment groups maintained the same net growth in shell dimensions as the ambient groups, which contradicts what was found by Harris et al. (1999) for the greenlip and blacklip abalone, the fact that the shells weighed less per mm of length indicates that they were either thinner or less dense following 4 weeks of exposure to seawater undersaturated with respect to aragonite. The noticeable difference in the appearance of the treatment shells also indicates that dissolution was occurring at least at the surface of the shells in the prismatic calcite layer. Since the treatment was always supersaturated with respect to calcite, this may indicate that the prismatic layer is comprised of both calcite and aragonite, contrary to what has been found previously in juvenile abalone (Verma et al., 2006). It is important to note that net growth and calcification rates were positive during the acidification period in both treatment groups; however, the lower rates of net calcification in the treatment group may suggest that the effect will likely be more pronounced and detrimental over increased magnitudes of conditions and with repeated upwelling events, as may be experienced in certain areas of California and in the near future during upwelling events due to the shoaling of the aragonite saturation state.

The tissue weights did not differ between treatment groups after the acidification period, which may indicate that the decreased DO and pH were not enough to impact the physiological health of the abalone. This contradicts what has been found in previous studies on brittlestars, whose muscle mass and function became impaired following positive net calcification in low pH (Wood et al., 2008). The treatment abalone showed significantly heightened activity over the ambient abalone during the first week of acidification. In the weeks following, the treatment groups followed the same basic trend and had the same activity levels. This could be interpreted
as a shock response when exposed to elevated $p$CO$_2$ and decreased DO that diminished once the abalone acclimated to the treatment. This observation needs further research for confirmation; however, it would indicate that abalone muscle function is not negatively affected by low pH as it was in brittlestars (Wood et al., 2008). The availability of food may have a great deal to do with the maintained health of the abalone during the experimental period. Food availability has been found to be directly correlated with abalone growth and health (Leighton, 1989). In nature, abalone would have to search and compete for food, as well as avoid predation, in order to stay alive and reproduce. Had there not been an excess of food in every aquarium the physical demands of maintaining positive net calcification in seawater undersaturated with respect to aragonite may have been more evident on a physiological level.

A very interesting result of this study is that the abalone that were exposed to the treatment for 4 weeks were able to recover to about the same weight gain rate as the abalone in the ambient aquaria once they were returned to ambient conditions. Although they were not able to completely recover the shell mass that was lost in the acidification period, treatment shells were only 4.2 mg (4%), lighter on average than ambient shells of the same length after the recovery period, as opposed to 5.9 mg (7%), lighter following the acidification period. Shells were also closer in appearance to the ambient shells after 3 weeks of ambient conditions, which may indicate the replacement of some of the aragonite that had dissolved in the acidification period. Calcium content in the treatment shells, though still lower than ambient shells of the same length, did not differ by as much following the recovery period.

These results indicate that abalone exposed to short periods of acidified seawater and decreased DO, such as would occur during an upwelling event, may only be impacted temporarily, and can recover to normal growth rates after conditions return to normal levels;
however, it is uncertain if the calcification rates can be accelerated to make up for the shell mass that was lost during the upwelling event. These findings show that the 3-week recovery period allowed for a slight narrowing of the difference between the treatment and ambient abalone, but not enough to mask the difference that was still significant between the treatment groups. Should abalone be exposed to several upwelling events before they are able to recover all lost shell mass, it is possible that their shells would become lighter and lower in calcium content following each event. In addition, impacts to juvenile abalone shells could be much worse if exposed to prolonged upwelling events, such as the two-month event recorded by Nam et al. (in press), that bring water undersaturated with respect to aragonite and low in DO into red abalone habitat.

It is still uncertain what will happen to red abalone if they are continuously exposed to upwelling conditions with high $pCO_2$ and low DO. The findings that juvenile red abalone, whose shells are mostly made up of calcite, have significantly lower net calcification in seawater undersaturated with respect to aragonite could have severe implications for larval and post-larval abalone, whose shells are made up almost entirely of aragonite. The results confirm the findings of most of the similar studies on other mollusks, which have found that either net calcification, growth, or reproduction are negatively affected by ocean acidification (Fabry et al., 2008; Widdicombe and Spicer, 2008; Dupont et al., 2010).

Decreased net calcification in nature may have severe repercussions on red abalone populations. The combination of a shoaling aragonite saturation horizon and upwelling could have significant impacts on a species that is already struggling to recover from severe depletion. If the findings of this study are apparent in juvenile red abalone, larval and post-larval stages may be at greater risk and should be studied to gain a more complete understanding of the future threats to the species. If research suggests that larval and juvenile abalone are unable to produce
adequate shells to protect them from predation, this could cause a decrease in the recruitment of abalone into the reproductively mature population. Abalone are broadcast spawners whose reproductive success is directly correlated to population density (Boolootian et al., 1962). A further reduction in recruitment may decrease successful breeding years, which are as rare as every 4 years in healthy populations (Karpov et al., 1998).

The findings of this study indicate that juvenile red abalone calcification is sensitive to the conditions of upwelling. Since high $pCO_2$ and low DO conditions have been found to decrease the net calcification rate of juvenile red abalone over short time periods, management efforts in areas predisposed to upwelling may need to be modified. For instance, marine reserves and seeding efforts should avoid areas subject to upwelling conditions that expose shallow waters to elevated $pCO_2$ and low DO. Seasons with increased upwelling, especially in La Niña years, should be avoided for seeding efforts in order to allow the abalone time to grow thicker shells before an upwelling event occurs. It would also be beneficial for seeding efforts to use abalone that are older and larger with thicker shells that may not be measurably impacted by seawater undersaturated with respect to aragonite. A great deal more research on younger red abalone stages exposed to varying magnitudes, frequencies, and durations of upwelling events should be conducted before the effects of ocean acidification and upwelling can be fully evaluated. In addition, the strength of shells exposed to upwelling conditions should be tested to quantify the force required to break or puncture the shell. Future research in this area should evaluate the response of other benthic invertebrates when exposed to upwelling conditions which have been exacerbated by ocean acidification. This information may aid in the adaptation of current management plans to incorporate projected changes in the ocean and the impacts it may have on benthic calcifying species.
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