THE EFFECTS OF OCEAN ACIDIFICATION AND REDUCED OXYGEN ON THE BEHAVIOR AND PHYSIOLOGY OF JUVENILE ROCKFISH

By

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A Thesis Presented to
The Faculty of Humboldt State University
In Partial Fulfillment of the Requirements for the Degree
Master of Science in Natural Resources: Fisheries

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May 2018

ABSTRACT

THE EFFECTS OF OCEAN ACIDIFICATION AND REDUCED OXYGEN ON THE BEHAVIOR AND PHYSIOLOGY OF JUVENILE ROCKFISH

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As climate change progresses, the frequency and duration of upwelling events that bring low pH, low dissolved oxygen (DO) water to nearshore habitats are expected to increase. In addition, long-term global changes in ocean pH and DO are expected to occur within the next few decades to centuries. Locally, there have been documented reductions in near-shore pH along with the expansion of oxygen minimum zones within the California Current System. However, very few studies have investigated the potential interactive effect of these stressors on temperate reef fish. For this thesis, two sets of laboratory experiments were conducted to determine the independent and interactive effects of reduced pH and DO on the behavior and physiology of juvenile rockfish (*Sebastes caurinus* and *Sebastes melanops*). Behavioral studies examined fish boldness using an escape trial and brain lateralization by testing the consistency of individual fishes' turning preference. Physiological studies measured critical swimming speed (U_{crit}), ventilation rate, standard metabolic rate (SMR), maximum metabolic rate (MMR), critical oxygen tension (P_{crit}), aerobic scope, and growth rate. Over the range of conditions examined, DO proved to have a much stronger effect on rockfish physiology than pH, suggesting that low-oxygen events may be more detrimental to individual fitness than ocean acidification. Significant effects of reduced DO on rockfish physiology include impaired swimming performance in *S. caurinus* and increased ventilation rate in *S. melanops*, but substantive responses in metabolic rates (e.g. SMR, MMR, aerobic scope, P_{crit}) were not observed. Juveniles of both species appear to be behaviorally resilient to exposure to reduced pH and DO. Together, these experiments address important questions regarding how temperate reef fish will respond to both the independent and interactive effect of these oceanographic stressors. Finally, they set the framework for studying species-specific susceptibility to pH and DO stressors.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Eric Bjorkstedt, for investing an incredible amount of time and energy in this project. He helped me with every aspect of this project, including study design, solving laboratory equipment issues, data analysis, and made numerous trips up to the marine lab at odd hours in the night to save the fish. Because of him, I am a better writer, more proficient in R, and overall, a more confident scientist.

I would like to thank Dr. Brian Tissot for serving on my committee and providing important advice on statistical analysis. I would also like to thank my other committee members, Dr. Tim Mulligan and Dr. Jeffrey Abell, for their support and advice throughout this project. Additionally, I appreciate all the help I received from our collaborators at CSU Monterey Bay, Moss Landing Marine Laboratories, and the Southwest Fisheries Science Center, including: Dr. Scott Hamilton, Dr. Sue Sogard, Dr. Cheryl Logan, Neosha Kashef, David Stafford, Evan Mattiasen, Jake Cline, and many others.

I am grateful for the help I received from the interns that invested a huge amount of time in this project, including: Evan Henricksen, Jeremiah Ets-Hokin, and Hana Busse. I am also thankful for assistance with fish collections from Roxanne Robertson, Brett Stacy, Franklin Moitzoa, Halle Shauer, and Kyle Weis. I also appreciate all the volunteers who helped with daily fish care, treatment monitoring, and laboratory trials including: Skylar Ritter, Aris Ownsbey, Caroline Hall, Allie Tissot, Sarah Wickman,

Riccardo Cicciraella, Jason Norris, and many others. I also thank the Trinidad Rancheria for permission to deploy SMURFs and collect fish in Trinidad Bay.

I appreciate all the help I received from HSU Boating Safety Officer Steve Monk and HSU Diving Safety Officer Rich Alvarez in support of fish collections. I also would like to thank the (now retired) Senior Lab Technician Dave Hoskins for an incredible amount of assistance in designing and building our laboratory setup, along with helping build several other pieces of equipment that were essential to this project, and Lab Technician Grant Eberle for assistance in the lab. Additionally, I would like to thank Marty Reed, who helped construct our brain lateralization double-T maze aquarium.

I would not have been able to do any of this without the love and support from my family, partner, and friends. In addition, a special shout out to Cashew Nut, for keeping me company during many long nights in the marine lab.

Funding for this project came from the National Science Foundation (Award #141697: “Collaborative Research: Ocean Acidification: RUI: Multiple Stressor Effects of Ocean Acidification and Hypoxia on Behavior, Physiology, and Gene Expression of Temperate Reef Fishes”), COAST, Marin Rod & Gun Club, and the Oliphant Scholarship.

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INTRODUCTION

Since pre-industrial times, atmospheric carbon dioxide (CO_2) levels have increased at an unprecedented rate, rising from 278 to over 400 ppm. The ocean acts as a major sink for CO_2; it has already absorbed 30% of the total anthropogenic CO_2 emitted since the Industrial Revolution (Sabine et al. 2004, Field et al. 2014). The rapid increase and absorption of CO_2 into the ocean is causing fundamental changes in the chemistry of seawater, resulting in ocean surface water to become more acidic, a process known as ocean acidification (OA) (Caldeira & Wickett 2003). The pH of ocean surface water has already dropped by 0.1 pH unit since pre-industrial times, resulting in a 26% increase in acidity (Field et al. 2014), and under continued 'business-as-usual' increases in atmospheric CO_2 concentrations, pH in surface waters is projected to decrease by an additional 0.3-0.4 pH units by 2100, making it more acidic than it has ever been in the past 400,000 years (Feely et al. 2004, Sabine et al. 2004, Caldeira & Wickett 2005).

Increasing atmospheric CO_2 concentrations are also expected to decrease dissolved oxygen (DO) concentrations in marine waters due to general warming of ocean surface water (Keeling et al. 2009, Shaffer et al. 2009, Helm et al. 2011, Long et al. 2016). This warming will increase water-column stratification, thus reducing oxygen ventilation across the pycnocline (Keeling & Garcia 2002). Over the next hundred years, it is predicted that global oxygen inventory in the ocean will decrease by 1-7% and that oxygen-minimum zones will continue to expand (Keeling et al. 2009, Shaffer et al. 2009, Cocco et al. 2013). Within the North Pacific basin, a 2°C increase in the upper 200-m is projected to occur from 1860-2100, with a concurrent decrease (18%) in dissolved oxygen by 2100. These changes will affect coastal ecosystems along the west coast of North America by changing the characteristics of source waters for coastal upwelling (Rykaczewski & Dunne 2010).

As climate change progresses, declines in ocean pH and dissolved oxygen (DO) content are expected to occur in concert and have major consequences for marine organisms, yet little is known about how fish will respond to the interactive effect of these oceanographic stressors (Chan et al. 2008, Feely et al. 2008, Pörtner 2008). It has been previously assumed that temperate fish that evolved in seasonal upwelling systems are less susceptible to the effects of OA and hypoxia (DO < 2.0 mg/L) due to natural exposure to variability in pH and DO. In these systems, wind-driven currents naturally bring relatively corrosive, low oxygen water from the depths to nearshore habitats, and under intense upwelling, can temporarily expose coastal ecosystems to conditions that match the reduced pH and DO conditions projected in the future as a consequence of ongoing anthropogenic CO_2 enrichment of the atmosphere (Feely et al. 2008, Booth et al. 2012, Frieder et al. 2012, Gruber et al. 2012).

In addition to global changes in ocean pH and DO, it is expected that ongoing climate change will intensify upwelling-favorable winds, however, the mechanism driving this change is still under active debate. One leading hypothesis is that global warming will cause an increase in land-sea temperature gradients, thus increasing upwelling favorable winds and thus upwelling intensity (Bakun 1990, Feely et al. 2008, Bakun et al. 2010, Bakun et al. 2015). An alternative mechanism for increased upwelling intensity includes a poleward shift in major atmospheric high-pressure cells (Rykaczewski et al. 2015). Regardless of the mechanism, several studies have provided evidence that upwelling intensity has and will continue to increase in many regions (Bakun 1990, Mendelssohn & Schwing 2002, Varela 2014, Bakun et al. 2015, Garcia-Reyes et al. 2015, Wang et al. 2015).

Specific to the California Current System (CCS), several studies have shown a change in source water being upwelled over the past 40 years (Snyder et al. 2003, Hauri et al. 2009, Garcia-Reyes & Largier 2010). In 2008, The North American West Coast Carbon Cruise observed an increase in the extent of corrosive upwelled water being brought to nearshore habitats along the west coast of North America, finding acidified water all the way to the surface in northern California coastal waters (Feely et al. 2008). Model simulations corroborate estimates that OA has already caused a 0.1 pH unit decrease in the CCS water since pre-industrial times (Hauri et al. 2009), and predict that by 2050, nearshore surface waters of the CCS will continue to decrease to pH 7.75 (Gruber et al. 2012).

An increase in upwelling-driven hypoxic events in the CCS have also been observed, causing large declines in nearshore dissolved oxygen content (Grantham et al. 2004, Bograd et al. 2008, Chan et al. 2008). Hydrographic data from the California Cooperative Oceanic Fisheries Investigations program (1984-2006) showed significant declines in oxygen and a shoaling of the hypoxic boundary within the Southern California Bight (SCB), consistent with hypothesized reductions in ocean ventilation due to increased warming and stratification (Bograd et al. 2008, Rykaczewski & Dunne

2010). Depleted oxygen conditions can have serious consequences for species distribution and home ranges. Off the coast of southern Oregon, home ranges of copper rockfish (*Sebastes caurinus*) were observed to decline by 33% during exposure to low oxygen waters (Rankin et al. 2013). In 2006, mass die offs and complete absence of fish off the coast of central Oregon (at least within a few meters of the seafloor) were documented during an unprecedented recording of anoxic (DO = 0 mg/L) conditions over the continental shelf (Chan et al. 2008). In the Southern California Bight (SCB), it is predicted that 55% of the habitat of cowcod (*S. levis*) (100-350 m depth range) will be affected by hypoxia within the next 20 years based on current trends in the shoaling of the hypoxic zone (McClatchie et al. 2010).

Since ocean pH and DO levels are often correlated, coastal organisms will likely be exposed to simultaneous and possibly synergistic, antagonistic, or additive stresses from concurrent reductions in pH and DO (Pörtner 2005, Melzner et al. 2013). However, the physiological tolerance of fish to simultaneous low-oxygen and acidic events remains poorly understood due to a lack of multiple stressor studies. While there is growing research on the interactive effects of temperature and reduced pH or temperature and reduced DO on fish physiology (Munday et al. 2009, Nowiki et al. 2012, McBryan et al. 2013), there is no published research, to date, that has investigated the interactive effect of reduced pH and DO on marine fish behavior or physiology.

Numerous studies and meta-analyses have reported negative effects of OA on shell formation and growth of marine calcifying organisms (Orr et al. 2005, Hoegh-Guldberg et al. 2007, Doney et al. 2009, Dupont et al. 2010, Kroeker et al. 2010). The response of marine fish to changes in ocean pH remains unclear, as lab experiments have shown high variability in response across species (Wittmann & Pörtner 2003, Kroeker et al. 2013, Heuer & Grosell 2014). However, it does appear that fish may be more susceptible to OA than previously assumed: 72% of fish species tested responded negatively to pH levels projected for the year 2100 (Wittmann & Pörtner 2003). Previous research has focused on neurosensory and behavioral responses in early life stages of tropical fish, but research on temperate species or long-term exposures has been lacking (Heuer & Grosell 2014). In early life stages of tropical species, reduced pH levels have been shown to disrupt brain lateralization (Domenici et al. 2011, Nilsson et al. 2012, Welch et al. 2014), affect auditory responses (Simpson et al. 2011), impair learning (Ferrari et al. 2012, Chivers et al. 2013), and alter perception of olfactory cues and thus disrupt fishes' ability to discriminate habitat, predators, prey, and other chemical cues (Munday et al. 2008, Dixson et al. 2010, Cripps et al. 2011, Nilsson et al. 2012, Welch et al. 2014). Recent research has shown reduced pH can also have a negative effect on the behavior of temperate fish species, such as longer escape times in stickleback (*Gasterosteus aculeatus*) (Jutfelt et al. 2013), increased anxiety in juvenile splitnose rockfish (*Sebastes diploproa*) (Hamilton et al. 2014), and reduced odor tracking ability in smooth dogfish (*Mustelus canis*) (Dixson et al. 2015).

In addition, exposure to reduced pH can cause physiological disruptions in fish such as reduced swimming performance and aerobic scope in juvenile copper rockfish (*Sebastes caurinus*) (Hamilton et al. 2017), reduced survival and growth in early life history stages of inland silverside (*Menidia beryllina*) (Baumann et al. 2011), changes in

RNA/DNA rations of Atlantic herring larvae (*Clupea harengus*) (Franke & Clemmesen 2011), negative effects on growth and metabolic rates of Senegalese sole larvae (*Solea senegalensis)* (Pimentel et al. 2015), and severe/lethal tissue damage in Atlantic cod larvae (*Gadus morhua*) (Frommel et al. 2012). However, reduced pH exposure intensity and duration varies greatly across studies, making species comparisons difficult.

Many of the observed alterations in fish behavior and physiology in response to reduced pH are thought to be caused by compensation from acid-base regulation. Exposure to reduced pH alters blood chemistry, and triggers an acid-base regulatory response to avoid acidosis by accumulating [HCO_3^-] with compensatory reductions in [Cl-] to maintain pH (i.e. [H+]). While fish can do this efficiently, there are several potential downstream effects linked to changes in the response/effectiveness of neurotransmitter receptors, specifically the neural gamma-aminobutyric acid type A ($GABA_A$) receptors, that govern chloride channels in neural and other cells and therefore affect the speed and strength of signal transmission in these cells (Nilsson et al. 2012). The general cost of coping with acid-base regulation can also effect several metabolic processes, with potential downstream effects on fish growth, reproduction, metabolic rate and swimming performance (Heuer & Grosell 2014). In some cases, OA has been demonstrated to increase standard metabolic rate (SMR), thereby reducing aerobic performance and overall animal fitness (Munday et al. 2009, Enzor et al. 2013). Acidification of the blood and respiratory pigments of tissues (from exposure to elevated pCO_2) has also been hypothesized to limit effective oxygen uptake and delivery, thus resulting in reduced maximum metabolic rate (MMR), which even further reduces an organisms aerobic scope (Munday et al. 2009, Heuer & Grosell 2014).

While several species have shown negative responses to reduced pH, other species appear to be resilient to reduced pH levels. Reduced pH levels have shown no effect on the aerobic scope of three tropical fish species (Couturier et al. 2013), swimming performance or aerobic scope of juvenile Atlantic cod (Melzner et al. 2009), hatch size or larval growth of walleye pollock (Hurst et al. 2013), or the swimming ability and growth of cobia larvae (Bignami et al. 2013). Contrary to prediction, exposure to reduced pH have enhanced aerobic capacity in the spiny damsel fish (Rummer et al. 2013) and increased otolith growth in white sea bass, cobia, and clownfish (Checkley et al. 2009, Munday et al. 2011, Bignami et al. 2013), which could have potential implications on auditory function (Simpson et al. 2011, Bignami et al. 2013). Even among species of similar life histories or ecologies, fish tolerance to reduced pH levels appear to be highly variable (Ferrari et al. 2011, Hamilton et al. 2017), proving that further research is needed.

The effects of oxygen depletion on fishes are better understood than the impacts from OA. Decreased oxygen levels have been found to negatively impact fish growth, reproduction, escape response, and increase mortality rates (Chabot & Dutil 1999, Pichavant et al. 2000, Landry et al. 2007, Domenici et al. 2015). Decreased DO levels have been found to negatively impact food intake and growth rate of Atlantic cod and juvenile turbot (Chabot & Dutil 1999, Pichavant et al. 2000), reduced activity level of common sole (Via et al. 1998), and impaired escape performance in golden grey mullet

(Lefrancois et al. 2005). The potential for OA to amplify or negate these responses warrants further research on the combined effect of reduced pH and DO on commercially valuable species.

Research Objectives

The objective of this study is to better understand the independent and interactive effects of reduced pH and DO on juvenile rockfish. In the CCS, rockfish are an abundant and diverse group of fish that inhabit a variety of ecological niches (Love et al. 2002). Rockfishes are a core component of the U.S. Pacific Coast fishery, with over 50 species managed by the Pacific Fisheries Management Council (Parker et al. 2000). Juvenile rockfish are a key prey source for many species of piscivorous fish (Merkel 1957, Petrovich 1970, Brodeur 1991), birds, and mammals (Mills et al. 2007). Due to life histories marked by slow growth and late maturation, rockfishes are susceptible to overfishing (Parker et al. 2000, Berkeley 2004). Furthermore, reproductive success is highly variable and sensitive to environmental and climate variability (Field and Ralston 2005, Ralston et al. 2013).

Settlement from pelagic to benthic/demersal habitats marks a sharp life history transition during which juveniles are at increased risk of predation. Successful juvenile recruitment is critical for replenishment of local populations, yet this stage is considered one of the most understudied aspects of rockfish life history (Love et al. 2002). Impediment of individuals' physiological or behavioral capacities during this transition period could greatly affect their survival probability, with consequences for overall recruitment to adult stocks (Dixson et al. 2012, Lima & Dill 1990). By using an integrative approach to assess individuals' responses to these stressors, this study aims to establish the groundwork for understanding how changes in ocean chemistry might affect temperate reef fish populations as atmospheric CO_2 concentrations continue to rise, and thus to provide critical information for fisheries managers as they look towards an uncertain and unprecedented future (Feely et al. 2008, Gruber et al. 2012).

Two sets of laboratory experiments were conducted to determine the independent and interactive effects of reduced pH and DO on the behavior and physiology of juvenile rockfish. The first experiment focused on the independent effects of reduced pH and DO on one species of rockfish. Four gradually reduced pH and DO levels were used to detect if changes in fish behavior and physiology increase with more extreme conditions and to detect if a physiological limit exists. pH treatment levels were based on current conditions and approximate global predictions for years ~2100, 2200, and 2300 (Caldeira & Wickett 2005). The second set of experiments aimed to investigate species-specific responses to the synergistic effects of pH and DO by exposing two species of rockfish with different early life history traits to independent and combined reduced pH and DO treatments. pH level was based on the global prediction for ~2200 (Caldeira & Wickett 2005). Both sets of experiments implemented a similar suite of tests, including behavioral assessments of fish boldness using an escape chamber and brain asymmetry (turning preference) and physiological assessments based on critical swimming speed (U_{crit}), ventilation rate, standard metabolic rate (SMR), maximum metabolic rate (MMR), aerobic scope, critical oxygen tension (P_{crit}), and growth rate. Together, these experiments address important questions regarding how temperate reef fish will respond to both the independent and interactive effect of these oceanographic stressors and the potential for species-specific susceptibility to pH and DO stressors.

Study species

The first set of experiments was conducted with copper rockfish (*Sebastes caurinus*), and the second set with both copper rockfish and black rockfish (*Sebastes melanops*). Black rockfish belong to the ‘structure schoolers’ or ‘water-column aggregators group’ that is characterized by an earlier parturition (Jan-Feb) and longer pelagic juvenile phase (4-6 months) (Love et al. 2002). They are often found in the lower third of the water column as recruits and juveniles (Carr 1991), and are one of the few rockfish species to utilize rocky intertidal tide pools as a nursery ground (Studebaker et al. 2011). As adults, they form mid-water schools, are associated with kelp or rock structures, and can occur to depths of over 350 m, although typically are found in shallower habitat (Lenarz et al. 1995, Love et al. 2002). Copper rockfish belong to the ‘nearshore demersal’ or ‘canopy’ group that is characterized by a shorter pelagic juvenile phase (1-3 months) and settle at smaller sizes (<2.0 cm) (Love et al. 2002). Copper rockfish recruit to kelp canopy and solitary adults are typically found in relatively shallow benthic habitats (up to depths of 183 m) compared to black rockfish (Lenarz et al. 1995, Love et al. 2002). Due to contrasts in early life history traits and potential exposure to different environmental conditions throughout their life history, including their larval and juvenile phases, these two species of rockfish might respond differently to pH and DO stressors.

Hypotheses

In this study, I tested several hypotheses focused on behavioral and physiological responses to exposure to reduced pH and reduced DO.

H_1: Exposure to reduced pH or reduced DO will affect behavioral patterns of juvenile rockfish, resulting in reduced boldness (longer escape times) and reduced bias in brain lateralization (decreased specialization).

H_2: Exposure to reduced pH or reduced DO will exert a negative effect on measures of physiological performance in juvenile rockfish, i.e., causing slower critical swimming speeds, greater ventilation rates, reduced aerobic performance, and slower growth rates.

H_3: Exposure to reduced pH and reduced DO will exert synergistic effects on fish boldness (longer escape times), brain lateralization bias, critical swimming speed, ventilation rate, aerobic performance, and growth rate.

H_4: Juvenile rockfish of species that settle in nearshore habitats at larger sizes and exhibit longer juvenile pelagic phases (e.g. black rockfish) are more resilient to the effects of reduced pH and reduced DO treatments than those that settle at smaller sizes and exhibit shorter juvenile pelagic phases (e.g. copper rockfish).

These hypotheses will all be tested against relevant null hypotheses (H_0) that exposure to reduced pH and DO has no effect on fish behavior or physiology.

MATERIALS AND METHODS

Ethics Statement

All collections and experiments conducted for this study conformed to the ethical guidelines of Humboldt State University Institutional Animal Care and Use Committee (permit #:13114.M.43-A) and the California Department of Fish and Wildlife (Scientific Collecting Permit #:13205).

Collection of Fish

Juvenile rockfish for laboratory trials were collected from coastal habitats in Trinidad Bay, CA (41.0593° N, 124.1431° W; Figure 1) using two SMURFs (Standard Monitoring Unit for the Recruitment of Fishes, Ammann 2004) deployed 1m below the surface on moored buoys. Free-divers retrieved SMURFs using BINCKE nets (Benthic Ichthyo-fauna Net for Coral/Kelp Environments, Anderson & Carr 1997), and brought them onboard a small zodiac, where fish were collected in buckets of seawater and quickly returned to land for processing (preliminary identification and measurement). Additional fish were captured by directly sweeping BINCKE nets through subsurface kelp canopy adjacent to SMURF sites. Collections took place between June-September in 2015 and 2016. All fish retained for laboratory trials were transported immediately to the Humboldt State University Marine Lab, and acclimated to laboratory conditions in holding tanks supplied with ambient seawater (~12°C, pH ~7.95, ~8.5 mg/L DO) and daily feedings of krill and mysid shrimp.

Figure 1. SMURF locations in Trinidad Bay, CA indicated by circles and Humboldt State University Marine Lab indicated by star. Fish were also collected in subsurface kelp beds adjacent to the SMURF arrays using BINKE nets.

Fish Tagging

Visible Implant Elastomer (VIE) Tags (Northwest Marine Technology) were used to identify fish and allow individual progress to be tracked through all experiments. Using a unique combination of body location and color, each fish was injected with a tag in 2 of 6 body locations (right or left: front, mid, or back; all dorsal) with 1 or 2 of four fluorescent colors (color could be repeated) (Figure 2). Fish were randomly assigned to a treatment tank after several weeks of acclimation to ambient laboratory conditions (pH ~7.95, 8.5 mg/L DO).

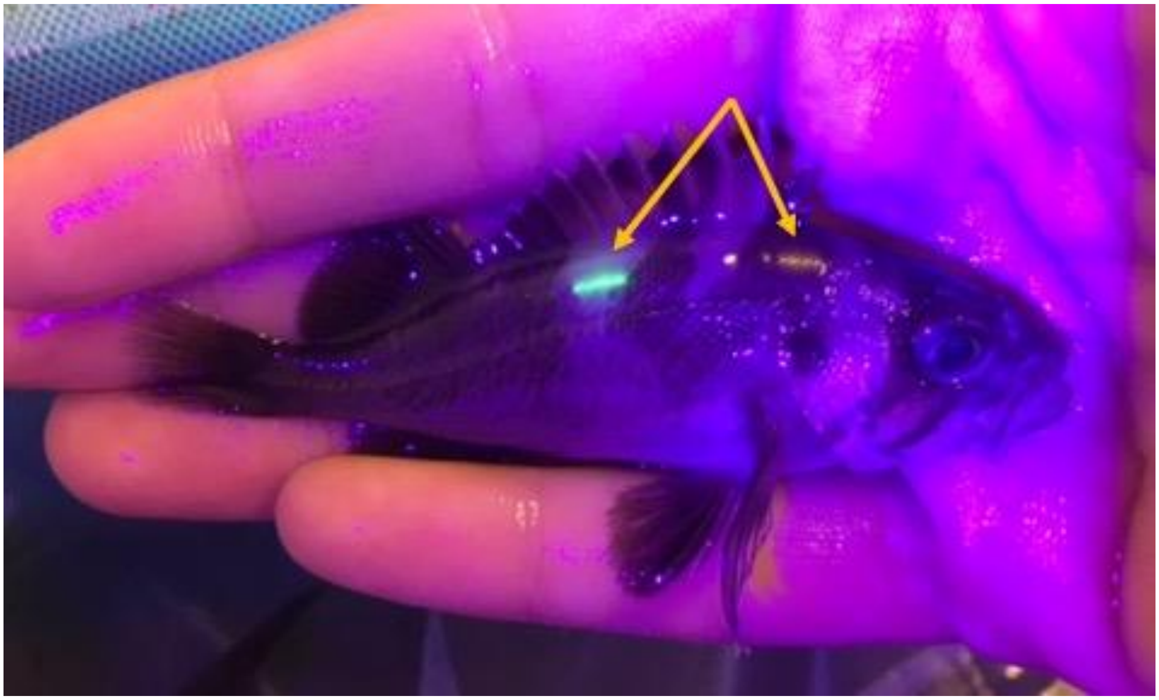

Figure 2. Example of fluorescent tags on juvenile rockfish in two body locations.

Experimental Infrastructure

Experiments were conducted by rearing juvenile rockfish in 80 L treatment tanks each of which was supplied seawater at a rate of approximately 10 mL/s from one of several 270 L reservoirs. Seawater supply entered each experimental treatment tank near the bottom driving continuous overflow from a drain port near the top of the tank. Overflow from experimental tanks was aerated before return to the lab water supply for subsequent filtering, aeration and recirculation. Reservoirs were automatically resupplied from the recirculating seawater system of the Humboldt State University Marine Lab.

Treatment conditions (pH and DO) were manipulated and continuously monitored in reservoir tanks (270 L), each of which supplied seawater to three 80 L treatment tanks, except for the control reservoir which supplied six treatment tanks during Year 1. Two treatment tanks from each reservoir were used for the experiments reported here; the third was used to house ‘reserve fish’ or for experiments not reported here. Each reservoir was fitted with two water pumps. One pump circulated water from the reservoir through manifolds from which a small fraction was diverted to treatment tanks and back to the reservoir. The other was oriented to drive vertical mixing within each cylinder to ensure water within the reservoir was well-mixed. Plumbing for supply and return to the manifolds was designed to allow continuous operation during temporary interruptions to seawater supply (e.g., during back-flushing of the lab’s sand filter) and to allow siphon-driven supply to the experimental tanks in the event of interruption of power to the pumps. Water temperature was maintained at approximately 12°C by placing all reservoirs in a temperature-regulated water bath controlled by an aquarium chiller (AquaEuro Systems).

Experimental conditions in treatment tanks were established and maintained by manipulating pH or DO in reservoirs that supplied the designated treatment tanks. Reductions in pH were achieved by bubbling CO_2 (industrial grade, Eureka Oxygen Supply) into the water to increase the amount of CO_2 in solution and thus drive changes in seawater pH. For pH treatments, each reservoir was equipped with a pH sensor with an integrated temperature probe (WTW pH 3310, Loligo Systems) through which pH and temperature was monitored at 1 s intervals. pH was monitored, logged, and controlled using Loligo Systems CapCTRL software for Windows. If pH deviated by +/- 0.01 pH units from the desired set point, the computer sent open (close) commands to the pH regulator (DAQ-M controller, Loligo Systems), which would open (close) the solenoid valve to inject (or stop injecting) CO_2 gas into the reservoir through an air diffuser. pH sensors were calibrated prior to installation and intermittently throughout the course of experiments using a three-point calibration (4, 7, 10 buffers). pH sensor offsets were accounted for in the CapCNTRL program.

Reductions in DO were achieved by bubbling a mist of pure nitrogen gas (N_2) (industrial grade, Eureka Oxygen Supply) into the water column of the reservoirs to strip oxygen out of aqueous solution. DO was monitored, logged, and controlled using the Loligo Systems WitroxCTRL software for Windows. For DO treatments, each reservoir contained a fiber optic mini sensor (Loligo Systems) connected to the DO regulator (Witrox 4, Loligo Systems). The Witrox 4 instrument included a temperature sensor, which was placed into one of the reduced DO reservoirs and used as a temperature proxy for the other reduced DO reservoirs. Oxygen readings compensated for changes in temperature, salinity and barometric pressure. Salinity parameter were adjusted as needed to match salinity measured in the laboratory flow-through seawater system that supplied the reservoirs (31-34 ppt), and barometric pressure was set constant to 1013 hPa. If DO levels deviated by +/- 0.01 mg/L from the desired set point, the computer sent open (close) commands to the digital relay device regulator (DAQ-M, Loligo Systems), which would open (close) the solenoid valve to inject (stop injecting) N_2 gas into the reservoir through an air diffuser. Oxygen sensors were calibrated prior to installment and intermittently throughout the course of experiments using a two-point calibration (0% air saturated water by bubbling N_2 gas into distilled water and 100% air saturated water by bubbling ambient air into distilled water).

Treatment tanks were fitted with lids and drain ports were installed to minimize head space and the potential for gas exchange with the atmosphere. Strip LEDs on a 12h:12h light:dark cycle were used to maintain a consistent photoperiod throughout the experimental period. Tanks were covered in black plastic to minimize visual disturbance of fishes and to reduce disruption of photoperiod due to lab lighting. Tanks were also equipped with artificial habitat (wrapped up plastic fencing weighted to the bottom of the tank) to provide security and a sense of structure. Treatment tank conditions (temperature, DO and pH) were checked regularly using handheld pH (pHC101, Hach) and DO (LDO101, Hach) probes. Treatment tank flow rates were calibrated regularly to 10 mL/s, which allowed a turnover rate of approximately 2.2 hours. Fish were fed commercially available krill every day in Year 1 and every other day in Year 2. Tanks were cleaned regularly using handheld nets or by siphoning out water and debris. Any fish discovered to have died were immediately removed from tanks, catalogued, and archived in a -80 C freezer.

Study Design: Year 1

In Year 1, I investigated the independent effects of exposure to reduced pH and reduced DO on juvenile copper rockfish behavior and physiology. Juvenile rockfish (n=140) were reared under seven different treatment conditions: three reduced pH treatments (target pH of 7.8, 7.5, 7.3), three reduced DO treatments (target concentrations of 6.0, 4.0, 2.0 mg/L), and a control (DO ~8.5 mg/L, pH ~7.95) based on the characteristics of water drawn from the recirculating laboratory seawater supply (Figure 3). Temperature was maintained at 12-13°C. Treatment conditions were replicated in two 80 L treatment tanks drawing from the same 270 L reservoir (n=10 fish/tank, 20 fish/treatment), resulting in a total of 14 treatment tanks. Behavioral trials (i.e. escape and brain lateralization) were completed for all fish. Physiology measurements (i.e. U_{crit}, SMR, MMR, aerobic scope, and P_{crit}) were conducted on a subset (n ~ 4) of copper rockfish from each treatment tank.

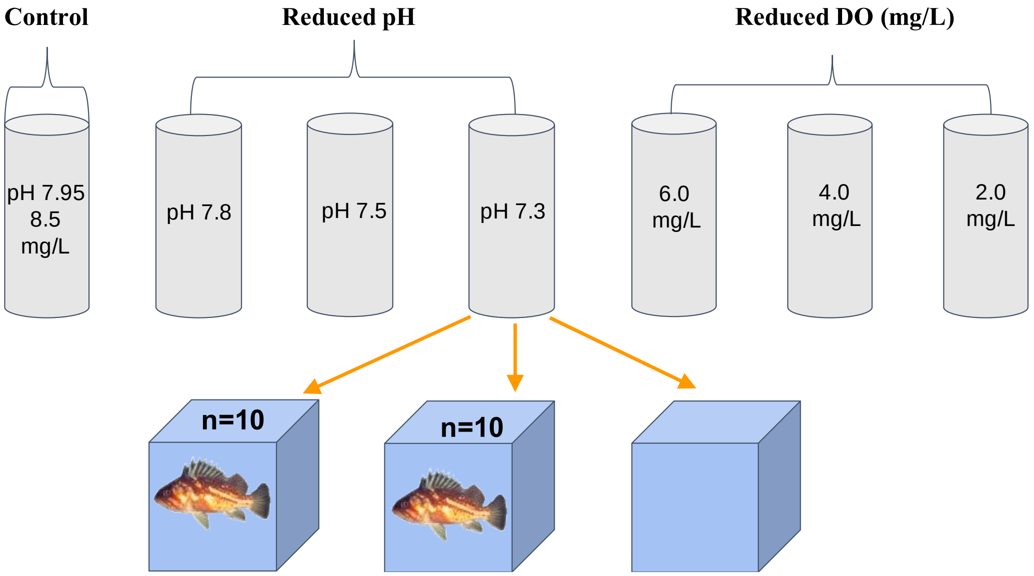

Figure 3. Year 1 laboratory experiment set-up. Each 270 L reservoir (grey cylinders) supplied three 80 L treatment tanks (blue squares). Two of the 80 L treatment tanks housed n=10 coppers. The third 80 L treatment tank in queue was used to house ‘reserve fish’ or for experiments not reported here. The control 270 L reservoir supplied six 80 L treatment tanks. Four of the 80 L control treatment tanks each housed n=10 coppers, while the other two 80 L control treatment tanks were used for experiments not reported here.

Study Design: Year 2

In the second year of this study, I investigated species-specific response to the independent and combined effect of reduced pH and DO on the behavior and physiology of juvenile black and copper rockfish. I used a 2x2-crossed design based on two pH levels (control and reduced pH treatment) and two DO levels (control and reduced DO treatment) that included four different treatment conditions: control (~pH 7.95, 8.5 mg/L DO), reduced pH (pH 7.5, 8.5 mg/L DO), reduced DO (~pH 7.95, 4.0 mg/L DO), and reduced pH*reduced DO (pH 7.5, 4.0 mg/L DO) (Figure 4). Each treatment was replicated in two reservoirs (270 L), and each reservoir supplied three 80 L treatment tanks (except for the control reservoir, which supplied six 80 L treatment tanks). Species were housed separately in replicate tanks for each treatment, one drawing water from each of the paired reservoirs for a given treatment (n=10 black rockfish/tank, n= 20/treatment; n=10 copper rockfish/tank, n=20/treatment). The third 80 L treatment tank in queue was used to house 'reserve fish' or for experiments not reported here. The same trials conducted in Year 1 were repeated in Year 2, with the addition of a ventilation rate physiology trial.

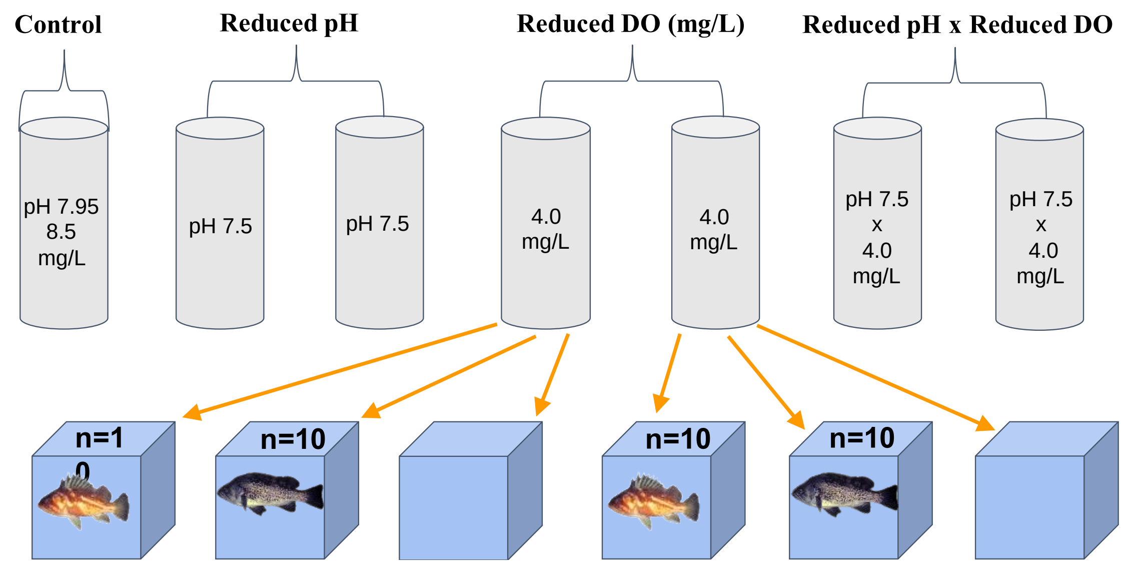

Figure 4. Year 2 laboratory experiment set-up. Each 270 L reservoir (grey cylinders) drained to three 80 L treatment tanks (blue squares). Copper and black rockfish were housed separately in treatment tanks (n=10/tank). The third 80 L treatment tank in queue was used to house 'reserve fish' or for experiments not reported here. The control 270 L reservoir supplied six 80 L treatment tanks. Two of the 80 L control treatment tanks each housed n=10 copper rockfish, two of the 80 L control treatment tanks each housed n=10 black rockfish, while the other two 80 L control treatment tanks were used for experiments not reported here.

Experimental Trials

Fish selected for experimental trials were introduced to treatment tanks, and pH or DO was adjusted over the course of 24-48 hours to allow a gradual acclimation to experimental treatment conditions. Exposure to full-strength treatment conditions continued for at least two weeks prior to the onset of trials. Fish were maintained in experimental treatment conditions during all trials by using the same treatment conditions in the experimental trial tanks that fish had been reared in. Several body reflexes (body flex, orientation, mouth closure, and dorsal fin erection) were tested prior to treatment introduction to ensure individuals were in good health (Depestele et al. 2014). All fish in this study were used in behavioral trials. Physiological experiments were conducted for a selected subset of fish (identity confirmed by tag) (n=4-5/tank; n=8-10/treatment). Fish were selected for physiology trials to maintain similar size distributions across all treatments within a species. The order in which fish were tested in trials was selected randomly. Prior to all trials, fish were collected with a hand net, identified by tag with the assistance of a UV light, measured to the nearest mm, and weighed (after blotting the tip of the tail to remove excess water drops) to the nearest 0.001g. After each trial, fish were immediately returned to their treatment tanks. Individuals were allowed to recover for at least five days between all experimental trials.

Behavioral trials

Escape response. Fish boldness was indexed by the amount of time it took a fish to escape from a chamber. Escape chambers were designed following Jutfelt et al. (2013) and Hamilton et al. (2017), and consisted of a white PVC cylinder (internal diameter = 10 cm, height = 35 cm) with a 5 cm diameter escape hole cut into one side, 7 cm from the base. A slit 8 cm from the top of the PVC cylinder allowed for the insertion and removal of a black Plexiglas divider. The chamber was placed within a 20-gallon black aquarium filled with treatment water. All sides of the aquarium were covered in black Styrofoam to minimize subjects' perception of the human observer. The observer stood out of view from the escape hole, and watched for the reflection of the fish exiting the chamber. A fish was considered to have escaped once its entire body had exited the escape chamber. In Year 2 trials, we included a "false bottom" in the escape chamber by placing a piece of white Styrofoam at the base of the escape hole to encourage fish to escape rather than to hide at the bottom of the escape chamber.

Fish were starved approximately 24 hours prior to the escape trials to maintain consistency in starvation period. Fish were placed into the top of the chamber with the Plexiglas divider in place, and allowed 15 minutes to acclimate. After acclimation, the divider was removed and the amount of time it took the fish to find and escape through the hole in the side of the chamber was recorded. If a fish had not escaped by 30 minutes, the experiment was terminated. All fish in experimental and control treatment were tested. In Year 1, escape trials were conducted on copper rockfish 2-3 weeks after exposure to treatment (n=140, copper rockfish). In Year 2, escape trials were conducted for 80 copper rockfish and 80 black rockfish. During Year 2, each individual was tested prior to being introduced to treatment conditions and again 2-3 weeks post-exposure to quantify changes in escape behavior post-exposure.

Brain lateralization. Brain lateralization is the tendency for some functions to be more specialized in the left or right side of the brain (Rogers 2002). Lateralization has been well established in most vertebrates, including fish (Bisazza et al. 1998, Rogers 2010). Left vs. right turning decisions were recorded to examine changes in brain lateralization using a detour test with a double T-maze after 3-4 weeks of exposure to treatment (protocols adapted from Domenici et al. 2011). The T-maze consisted of Plexiglas partitions 30 cm long X 10 cm wide X 10 cm high on each side of ~80 L tank, creating a 10 cm wide corridor down the middle. Fish were starved approximately 24 hours prior to experiment. Fish were placed into the corridor of the maze and given a 3-minute acclimation period. Following acclimation, the subject was coaxed towards the end of the maze using a plastic rod, and the direction it turned once it reached the end of the T-maze was recorded (a turn was designated as >45° change in orientation of their head in either direction). Each fish was tested 10 times, with an equal number of trials in each direction to account for potential effects of asymmetrical maze construction or subjects' response to the presence of the experimenter.

Relative lateralization index (L_R), or population-level lateralization, measures whether the mean response across individuals in a group is left- or right-biased in turning direction (Bisazza et al. 1997). The relative lateralization index is calculated using the following equation:

$$L_R = [[\#Right\ Turns - \#\ Left\ Turns] / [\#\ Right\ Turns + \#\ Left\ Turns]] * 100$$

This index ranges from -100 to 100, where L_R = -100 indicates all left-turns, L_R = 100 indicates all right-turns, and L_R = 0 indicates equal turns in both directions. However, while a population sample might not be biased in either direction, individuals within that sample might themselves be left- or right-biased. Taking the absolute value of L_R produces the absolute lateralization index (L_A), which can be used to compare lateralization strength (irrespective of turning direction) across groups at the individual level. The L_A index ranges from 0 (individual turned in equal proportions to left and right) to 100 (individual turned in same direction on all trials). Both L_R and L_A were calculated and reported in this study.

Physiology trials

All physiological responses, except for ventilation rate, were scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship to account for variability in temperature during experimental trials. The slope for temperature dependence of respiration (Q_{10}) was based off several scorpaenid respiration studies (Boehlert et al. 1991, Yang et al. 1992, Kita et al. 1996, Vetter & Lynn 1997, Harvey 2005). Ventilation rate was not scaled for temperature because continuous temperature measurements were not recorded during ventilation trials.

Critical swimming speed (U_{crit}). Swimming performance was quantified by estimating the critical swimming speed (U_{crit}) for individual fish after 4-8 weeks of acclimation to treatment conditions. U_{crit} is a standardized protocol that has been effectively used to estimate maximal swimming performance in fish (Brett 1964, Tierney 2011). I used a 30 L swim tunnel (test section: 55x14x14 cm, footprint: 147x53 cm; Loligo) and a propeller attached to an external motor to control water velocity. The relationship between the analog output on the motor (rotations per second, Hz) and flow velocity in the swim tunnel (m/s) was quantified using a hand-held digital flow meter (flowtherm NT, Höntzsch); this relationship was applied to determine appropriate motor settings for scaling flow in the tunnel to individual's body length (BL).

Fish selected for trials were starved for approximately 24 hours prior to the trial. At the start of a trial, a fish was placed into the middle of the test section of the swim tunnel facing forward and given 15 minutes to acclimate at a flow speed of ½ BL/s. Following acclimation, the flow speed was increased by one body length per second every two minutes until the fish became fatigued (could no longer maintain swimming position for entire two minutes). If the fish rested against the back grate, the observer used a flashlight to motivate the fish to continue swimming. If the fish continued to rest, the observer stopped the timer and reversed the current to push the fish away from the rear grate, then re-established the original direction and strength of flow to encourage the fish to swim again. After the fish rested against the back grate for a third time, it was considered fatigued and the trial ended.

U_{crit} was calculated using the following equation:

$$Ucrit = Ui + U \times (\frac{t}{ti})$$

where U_i = highest velocity maintained during the trial, U = velocity increment (1 BL/sec), t = time elapsed at fatigue velocity (in seconds), and t_i = set time interval for each increment in velocity (120 s) (Fisher et al. 2000, Bellwood & Fisher 2001, Kashef et al. 2014). During both years, n=4-5/tank; n=8-10/treatment were tested.

Ventilation rate. Ventilation experiments were only conducted during Year 2. Fish selected for trials were starved for approximately 24 hours prior to the trial. To measure ventilation rate, fish were assigned randomly to one of 10, 12x6x6 cm cell, ventilation chambers. Reservoirs of treatment water were used to provide a continuous supply to each chamber cell at approximately 5 mL/s. Two GoPro video cameras were mounted above the chambers so that all 10 chambers were visible in their field of view. A blind made from white waterproof paper was placed over the chamber to minimize disturbance to fish, and fish were allowed to acclimate for two hours. Following acclimation, the GoPro cameras were turned on remotely (using the GoPro iPhone app) to record for 30 minutes. Video was shot at a medium recording angle, 60 fps, and 1080i resolution. At the end of the experiment, fish were returned to their treatment tanks.

Ventilation rate was quantified from recorded video. The number of operculum openings for each fish was counted during three separate one-minute time intervals during the 30-minute recording. Target time intervals included 05:00-06:00, 15:00-16:00, and 25:00-26:00. If operculum movements for an individual were not continuously visible in the video frame during any of these time intervals, the window of observations was shifted to achieve a complete minute of observations (e.g. 5:15-6:15). Counts from the three one-minute periods were averaged as a measure of ventilation rate (number of operculum openings/minute).

Standard metabolic rate (SMR). Standard metabolic rate is defined as the oxygen uptake rate of a post-absorptive (fasting), inactive organism and is considered the minimum metabolic rate required to sustain life (Fry 1971). SMR was measured using an automated intermittent-flow respirometry system, which is considered the preferred method for estimating oxygen consumption and aerobic scope for aquatic organisms (Svendsen et al. 2016). Fish were starved 48 hours prior to trials. The respirometry system consisted of four horizontal respirometry chambers (128, 358, or 650 mL) immersed in a common bath. Each chamber was connected to a pair of pumps: one plumbed to allow the chamber to be flushed with water from the bath, and the other to recirculate water in the chamber in an isolated loop. Fiber optical oxygen sensors (Year 1: dipping probe oxygen mini sensors, Loligo Systems; Year 2: flow-through oxygen mini sensors, Loligo Systems) sampled oxygen concentration in the recirculating loop every second, which is active during both flush and closed-loop conditions. All oxygen sensors were calibrated using a two-point calibration (0% air saturated water by bubbling N_2 gas into distilled water and 100% air saturated water by bubbling O_2 into distilled water). An oxygen sensor and feedback control that governs N_2 bubbling were used to regulate DO in the bath. In Year 1, a WTW pH sensor and feedback control (CapCNTRL program) coupled to a CO_2 supply were used to control pH (described in Methods: Experimental Infrastructure). In Year 2, pH was controlled using an Apex AquaController System (Neptune Systems). The Apex pH sensor was calibrated using a two-point calibration (7 and 10 buffers), and included a separate temperature probe to compensate for changes in temperature.

DO measurements and control of DO was automated using Loligo Systems AutoResp software coupled to the oxygen sensor and Witrox 1 instrument. AutoResp was set to continuously cycle through 300-second flush, 300-second wait, and 300-second measurement phases throughout the trial (at least 12 h overnight). The wait phase was reduced to 60 seconds during Year 2, to reduce exposure to severely depleted DO. The wait period allowed for sufficient recirculation within the respirometry chamber to stabilize the decline in oxygen concentration over time prior to data collection during the measurement phase.

Water bath temperature was maintained at approximately 12°C by circulating bath water through a stainless-steel coil immersed in a separate ice-bath. Salinity settings (31-34 ppt) were based on measurements of the laboratory water supply, and barometric pressure parameter was maintained at 1013 hPa in AutoResp for all trials. A pump was placed in the center of the bath to keep the ambient bath water well circulated. The bath was covered with a black plastic tarp for the duration of the trial to minimize disturbance to fish. A total of n=4 fish/tank; n=8 fish/treatment were tested for SMR trials. A schematic of the respirometry set-up and design is shown in Figure 5.

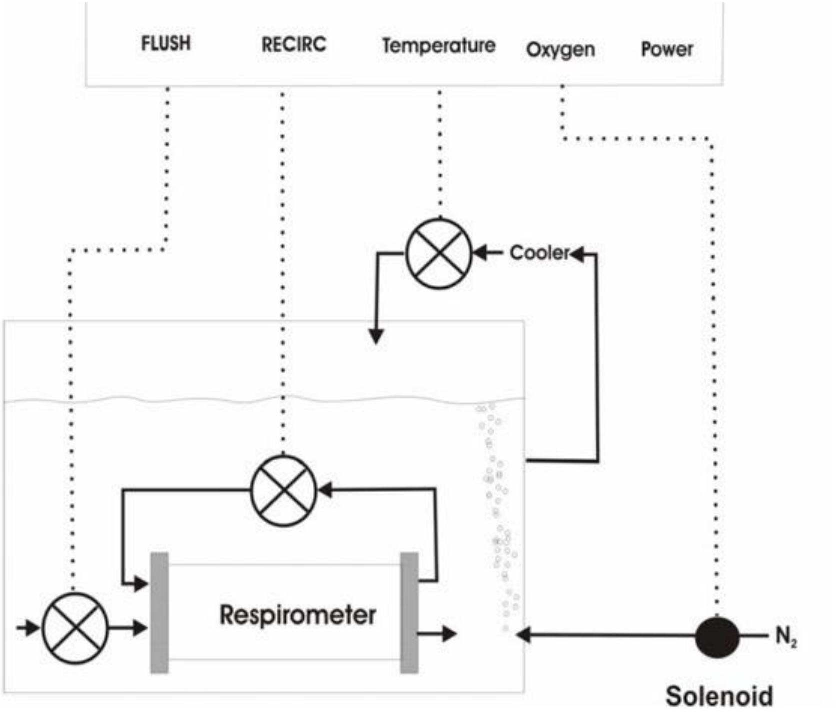

Figure 5. Respirometry design for SMR, MMR, and *P_{crit}* experiments. Each respirometry chamber was connected to a flush and recirculating pump. The flush pump flushed water from the external bath through the respirometer and back into the ambient bath. The outlet hose of the flush pump was placed above the water surface to avoid siphon water from entering the respirometer when the flush pump was turned off. The recirculating pump flowed water through the respirometer and oxygen sensor unit. During Year 1, dipping probe oxygen sensors were used (Loligo Systems). During Year 2, flow-through mini sensors that were integrated into a t-shaped flow-through cell were used. The flow-through cell connected to the Oxy-4 sensor through a polymer optical fiber that consists of a 2mm inner diameter glass tube coated with oxygen sensor. Water bath oxygen level was manipulated and controlled by bubbling N_2 gas directly into the bath. Water bath pH was manipulated by bubbling in CO_2, controlled via CapCNTRL in Year 1 and Apex System in Year 2. Respirometry chamber oxygen sensors connected to the Oxy-4 instrument, a four-channel oxygen instrument for measuring changes in DO. The water bath oxygen sensor and temperature probe connected to the Witrox 1 instrument. An external ice-filled cooler with a cooling coil was used to maintain temperature in the respirometry cooler. Tubing was minimized to limit volume and measured for inclusion in volume estimates.

Calculating SMR. The rate of fish respiration was measured from the decline in oxygen concentration in the chamber during each 300-second measurement phase. Metabolic rates of fish were initially high due to handling stress, but were observed to stabilize after a few hours as fish acclimated to chambers. Since SMR trials lasted >12 hours, >36 MO2 values were calculated per individual. Estimates of oxygen consumption during each measurement phase were retained for subsequent analysis if the relationship between oxygen concentration and time exhibited a strong goodness-of-fit ($r^2 > 0.9$, confirmed by visual inspection of fit and residuals). MO2 was estimated for each measurement phase using the following equation:

$$MO_2[mgO_2/kg*hr] = slope[mg\ O_2/l*hr] * (Resp.\ volume[ml] \ /\ wet\ weight[g])$$

Estimates were subsequently adjusted (using $Q_{10} = 2$) for differences in temperature (from a standard temperature of 12°C) during the measurement, using the following equation:

$$R_2 = R_1 {Q_{10}}^{(T_1 - T_1)/10℃}$$

where R_2 = temperature adjusted MO_2, R_1 = estimated (unadjusted) MO_2, $Q_{10} = 2$, T_1 = temperature reading during MO_2 measurement, T_2 = 12°C. The mean of the five lowest (temperature-corrected) MO_2 estimates was taken as the estimate of SMR.

Maximum metabolic rate (MMR). Maximum metabolic rate (MMR) is the maximum rate at which an organism can consume oxygen from the environment (Fry 1971). MMR was measured using the ‘chase’ method (Clark et al. 2013). Following a 48-hour fast, fish were placed into the same swim flume used for U_{crit} measurements, forced to swim continuously for five minutes at one body length below their critical swimming speed (fish were chased with a stick if they attempted to rest), and immediately transferred into a respirometry chamber (see SMR methods). Oxygen consumption was measured over a single 300-second measurement period in respirometry chambers used for SMR. To minimize recovery from exercise, no flush period preceded measurement. Since the velocity for MMR trials was based on individual performance during U_{crit} trials, fish that demonstrated high swimming capabilities in U_{crit} trials were chased at higher velocities for MMR trials.

Calculating maximum metabolic rate (MMR). Maximum metabolic rate (MMR) was calculated using the same equation used to calculate SMR (temperature corrected). Only one MO_2 measurement was calculated per fish, since additional measurement loops would provide the fish too much time to rest post-exercise.

Aerobic scope. Aerobic scope is the difference between minimum and maximum oxygen consumption rate, and is considered a metric of whole animal fitness (Fry 1971). Aerobic scope was calculated by taking the difference between MMR and SMR for each individual fish.

Critical oxygen level (P_{crit}). Critical oxygen level (P_{crit}) is used as a measure of hypoxia tolerance, and is the oxygen level threshold below which a fish can no longer regulate oxygen uptake and oxygen uptake begins to decrease linearly with declines in ambient pO_2. Below this oxygen threshold, fish transition from aerobic to anaerobic respiration (Rogers et al. 2016). P_{crit} was calculated using intermittent-flow respirometry with step-wise reductions in ambient DO. P_{crit} was estimated the morning following the SMR trial (after fish had acclimated for >12 hours in respirometry chambers). For all fish, regardless of treatment level, DO was set to 70% O_2 saturation for initiation of P_{crit} measurement series and fish were allowed to acclimate for one hour. Beginning at 70% O_2 saturation (~6.5 mg/L), three cycles of flush, wait, and measurement phases (same duration used in SMR) were completed. Then, oxygen saturation was stepped down by 10% for another three cycles. This pattern was repeated until a set of trials at 40% O_2 saturation (~3.0 mg/L) was completed, at which point O_2 saturation was reduced by 5% for each subsequent set of measurements until oxygen levels reached 10-15% O_2 saturation (~0.8-2.0 mg/L). Experiments ended once the decline in oxygen uptake was clearly resolved, i.e., a decline was observed over at least three levels of ambient pO_2, which generally occurred by the time pO_2 had been reduced to 15% O_2 saturation (~2.0 mg/L). At the end of the experiment, fish were immediately returned to treatment tanks and monitored for recovery. A total of n=4 fish/tank (n=8 fish/treatment) were tested for P_{crit} trials. Temperature and pH during P_{crit} trials was maintained using the same procedures used in SMR.

After each SMR/P_{crit} trial, the entire bath of water in the cooler was drained, the chambers, bath, and pumps were rinsed with filtered freshwater, which was subsequently drained before being refilled with seawater for the next SMR/P_{crit} trial. After every third SMR/P_{crit} trial, the entire system was thoroughly disinfected by soaking/circulating a strong solution of bleach in freshwater, and rinsed several times prior to initiation of the next set of trials.

Calculating critical oxygen saturation (P_{crit}). Using the R package “segmented” (Muggeo 2008), piecewise linear regression was used to fit a broken-stick model relating metabolic rate to ambient oxygen concentrations (Rogers et al. 2016, Monterio et al. 2013). The critical oxygen level (P_{crit}) was determined as the intersection between the regression line fitted through MO2 estimates at oxygen concentrations above P_{crit} and fitted least squares regression through data points that show a progressive decline in MO_2 (i.e., at oxygen concentrations below P_{crit}). The oxygen level corresponding to the ‘breakpoint’ was taken as the estimate of P_{crit}. An example P_{crit} estimation is shown in Figure 6. It is recognized that some uncertainty exists in the slope estimated for oxygen consumption rate used for metabolic measurements, as well as uncertainty in the measurement of dissolved oxygen in the chamber at the time those measurements were taken. Only those estimates with strong goodness of fit were chosen (r^2 >0.9), thus it is assumed that uncertainty in the estimates of metabolic measurements has minimal influence on estimation of P_{crit}.

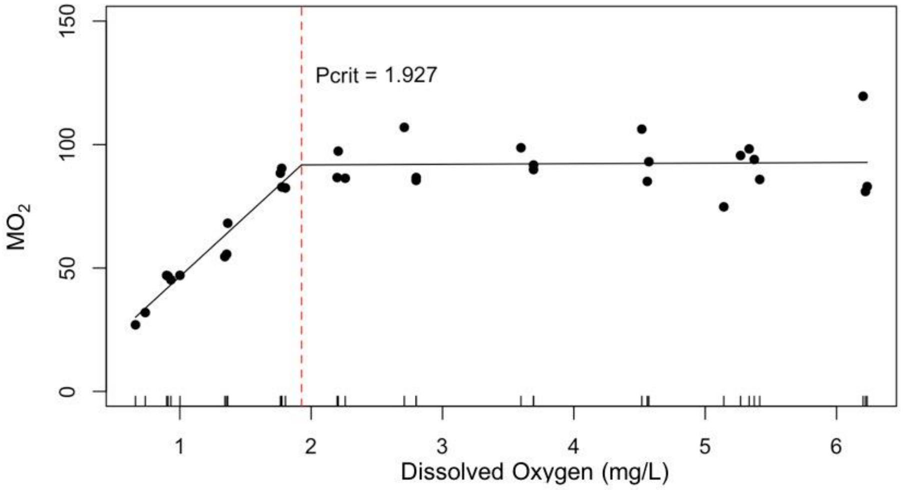

Figure 6. *Pcrit* calculation example using R package “segmented”. Red dashed line is at the critical oxygen level; the intersection between the regression line fitted through MO_2 estimates at oxygen concentrations above *Pcrit* and fitted least squares regression through data points that show a progressive decline in MO_2.

Somatic growth. Somatic growth rates were calculated by dividing change in total length and weight by the number of days in treatment (mm/day, g/day). For Year 1 fish, changes in somatic growth rate were measured for a subset of fish (n=6-10 fish/treatment) prior to treatment initiation and prior to U_{crit} trials (57-69 days after exposure to treatment). Year 1 copper rockfish somatic growth rate was also measured for all individuals that survived to the end of the experiment (223-276 days of exposure to treatment). For Year 2 fish, somatic growth rate (mm/day) was measured for a subset of individuals (n=8 fish/treatment) prior to treatment and prior to U_{crit} trials (59-73 days after exposure to treatment). Since there were high mortalities and malnutrition issues towards the end of the experiment, somatic growth was not measured for fish that survived to the end of the experiment.

Statistical analysis

Experimental design consisted of two tank replicates for each treatment condition (including control). Since responses of individuals within the same tank cannot be regarded as independent from each other, the issue of non-independence was resolved by using linear mixed effect models. By including a random effect for tank, any potential tank effect was accounted for by assuming a different baseline response for each tank. All statistical analysis was conducted using the statistical software language R (Version 1.0.143, RStudio Team 2015) using packages “nlme” and “lmerTest” (Faraway 2006, Pinherio et al. 2014).

Covariates in the linear mixed effect model included total length to account for variability in individual fish size, and tank-specific experimental conditions (pH, DO, and temperature) that were measured before or during each experiment to account for any deviation from the nominal (but slightly variable) conditions established in the treatment tanks. For analyses on SMR, MMR, aerobic scope, and P_{crit}, fish wet weight was included as a covariate instead of total length. Tank-specific experimental conditions were sequentially removed using backwards selection if their influence proved insignificant ($P > 0.05$). Fish size was retained in all models, regardless of statistical significance. Outliers were included in all statistical analyses.

For Year 1 results, linear mixed effect models were used to independently test the difference between control and treatment (reduced pH or reduced DO treatment) means on copper rockfish behavioral (e.g. escape, brain lateralization) and physiological (e.g. U_{crit}, SMR, MMR, P_{crit}, aerobic scope, somatic growth) responses, and included tank as a random effect. For Year 2 results, linear mixed effect models with an interaction term were used to assess the effect of reduced DO, reduced pH, and the interaction between both stressors on juvenile copper and black rockfish behavioral (e.g. escape, brain lateralization) and physiological (e.g. U_{crit}, ventilation, SMR, MMR, P_{crit}, aerobic scope, somatic growth) responses, and included tank as a random effect. Model assumptions, such as homogeneity of variance and normally distributed residuals, were visually assessed using residuals vs. fitted and QQ plots. Additionally, Levene's tests (using R package "leveneTest") were conducted on all models to assess the homogeneity of variance assumption. Year 1 escape response was log-transformed to better fit model assumptions. In all other cases, log-transformation did not improve model fit, and thus response variables were kept on their original scale. The R package "multcomp" was used for making post-hoc Tukey comparisons on models that demonstrated a significant treatment effect (Hothorn et al. 2017).

Log-likelihood ratios were used to test the significance of the random tank effect, by comparing the log-likelihood of the model with and without tank included as a random effect. Regardless of tank significance, it was retained in all models due to experimental design and provided useful information for future experiments (Faraway 2006). Statistical significance was evaluated at $\alpha = 0.05$.

RESULTS

Treatment Conditions

Treatment conditions during both years were relatively stable and remained near the desired set points. Under normal operations, conditions in reservoirs would fluctuate over time due to less-than-instantaneous feedback in the control software, but this variability was attenuated by the limited rate of supply from reservoirs to treatment tanks and correspondingly slow dilution in the experimental tanks (Figure 7). Water in experimental tanks had a residence time of approximately 2.2 hours.

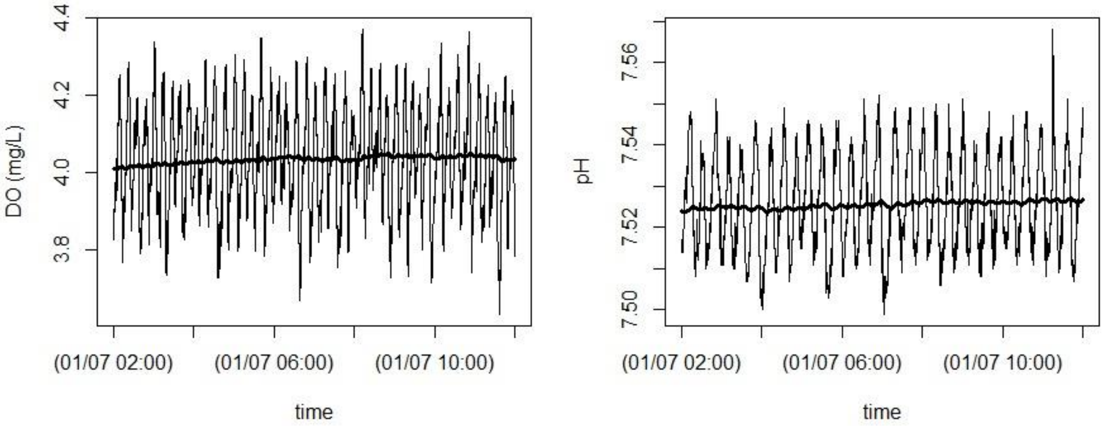

Figure 7. Left panel: Example of reservoir-tank variability in dissolved oxygen (mg/L) conditions (over ~10 hours). The thick line indicates expected dissolved oxygen conditions based on simple dilution model for flow-through treatment tanks based on 10 mL/s into an 80 L (80,000 mL) tank. Right panel: Example of reservoir-tank variability in pH conditions (over ~ 10 hours). The thick line indicates expected pH conditions based on simple dilution model for flow-through treatment tanks based on 10 mL/s into an 80 L (80,000 mL) tank.

On several occasions, disruptions to reservoir controls (e.g., disruption of USB communications between pH sensors and the master computer, uncontrolled computer shut downs, power outages) led to brief exposures to more extreme levels of pH or DO (due to excess CO_2 or N_2 bubbling, respectively) or allowed treatments to revert to control conditions. Departures from target conditions in the reservoirs were typically resolved within an hour or two, preventing extensive, long-term disruption of experimental conditions. In rare cases of more extreme (more stressful) conditions developing in the reservoirs, water supply to treatment tanks was restricted or shut down, and reservoirs were partially emptied and refilled to rapidly restore treatment conditions. Following more stressful events, fish were allowed ample time for recovery or re-acclimated to treatment prior to trials. Treatment tank conditions were spot checked regularly; average conditions are listed in Tables 1-2.

Table 1. Mean treatment tank conditions (pH, DO, and temperature) and standard deviations for copper rockfish during the first experiment. Measurements were taken regularly from September 29, 2015 to July 28, 2016 using a handheld pH and DO probe (PH101, LDO10; Hach).

Treatment	*Tank ID*	*pH*	*DO*	*Temperature*
Control	1	7.94 +/- 0.03	8.44 +/- 0.19	12.40 +/- 0.23
	2	7.95 +/- 0.03	8.47 +/- 0.16	12.34 +/- 0.22
pH 7.8	1	7.83 +/- 0.05	8.42 +/- 0.17	12.49 +/- 0.31
	2	7.82 +/- 0.05	8.37 +/- 0.24	12.49 +/- 0.33
pH 7.5	1	7.53 +/- 0.09	8.38 +/- 0.18	12.57 +/- 0.27
	2	7.55 +/- 0.09	8.41 +/- 0.24	12.53 +/- 0.32
pH 7.3	1	7.29 +/- 0.11	8.33 +/- 0.59	12.56 +/- 0.33
	2	7.29 +/- 0.12	8.36 +/- 0.34	12.58 +/- 0.34
DO 6.0	1	7.95 +/- 0.10	6.22 +/- 0.47	12.78 +/- 0.29
	2	7.95 +/- 0.08	6.13 +/- 0.46	12.78 +/- 0.31
DO 4.0	1	7.94 +/- 0.04	4.44 +/- 0.72	12.66 +/- 0.32
	2	7.95 +/- 0.03	4.47 +/- 0.70	12.51 +/- 1.25
DO 2.0	1	7.96 +/- 0.02	2.73 +/- 1.24	12.82 +/- 0.27
	2	7.95 +/- 0.03	2.55 +/- 1.00	12.71 +/- 0.27

Table 2. Mean treatment tank conditions (pH, DO, and temperature) and standard deviations for copper and black rockfish. Measurements were taken regularly from October 11, 2016 to June 16, 2017 using a handheld pH and DO probe (PH101, LDO10; Hach).

Treatment	*Species*	*Tank ID*	*pH*	*DO*	*Temperature*
Control	Copper	1	7.94 +/- 0.03	8.65 +/- 0.19	12.10 +/- 0.41
		2	7.93 +/- 0.03	8.60 +/- 0.17	12.13 +/- 0.43
pH		1	7.51 +/- 0.08	8.58 +/- 0.19	12.35 +/- 0.46
		2	7.51 +/- 0.06	8.53 +/- 0.25	12.57 +/- 0.54
DO		1	7.93 +/- 0.03	4.14 +/- 0.17	12.51 +/- 0.50
		2	7.91 +/- 0.04	4.33 +/- 0.26	12.59 +/- 0.50
*pH*DO*		1	7.48 +/- 0.08	4.38 +/- 0.30	12.51 +/- 0.53
		2	7.50 +/- 0.06	4.34 +/- 0.15	12.51 +/- 0.51
Control	Black	1	7.94 +/- 0.03	8.65 +/- 0.19	12.10 +/- 0.41
		2	7.93 +/- 0.03	8.60 +/- 0.19	12.13 +/- 0.43
pH		1	7.49 +/- 0.08	8.49 +/- 0.26	12.35 +/- 0.45
		2	7.51 +/- 0.05	8.54 +/- 0.22	12.54 +/- 0.54
DO		1	7.93 +/- 0.03	4.17 +/- 0.23	12.47 +/- 0.50
		2	7.93 +/- 0.04	4.22 +/- 0.36	12.56 +/- 0.49
*pH*DO*		1	7.50 +/- 0.06	4.48 +/- 0.34	12.45 +/- 0.53
		2	7.50 +/- 0.06	4.28 +/- 0.25	12.52 +/- 0.53

Tank Effects

A significant tank effect was not detected in any analysis of behavioral or physiological responses to reduced pH or DO. Thus, tank effects are not reported or discussed further in the results.

Assessment of Homogeneity of Variance

Levene’s tests were conducted on all models in this analysis to assess the homogeneity of variance assumption. In all cases, the Levene’s test proved to be insignificant, suggesting that differences in variances across treatment groups is not significant. However, due to the low sample size used across trials, it is acknowledged that the power to detect a significant difference in variance across treatments is low.

Fish Characteristics

Fish size distributions remained consistent across treatment groups throughout the series of experimental trials (Appendices A-C). In most cases, an effect of fish size on behavioral or physiological response variables was not detected. Exceptions to this pattern were observed for brain lateralization in copper rockfish exposed to reduced DO (total length) and to various measures of oxygen consumption in fish exposed to reduced pH and DO (weight). Size effects, regardless of significance, are presented for all analyses.

Experimental Tank Seawater Conditions

Seawater conditions (e.g. pH, DO, and temperature) were measured in experimental tanks (e.g. escape chamber, double T-maze, swim flume, ventilation chambers, respirometry chambers) prior or during each trial to account for any deviation from the treatment conditions fish had been acclimated to in their treatment tanks. In nearly all cases, an effect of experimental tank seawater conditions on behavioral or physiological response variables was not detected and is not reported in the results. An exception to this pattern was observed for temperature, which demonstrated a significant effect in the maximum metabolic rate of black rockfish exposed to reduced pH and DO, despite MMR for black rockfish being scaled to a standardized temperature using a Q_{10} (=2) relationship (reviewed in greater detail below).

Fish Health

During Year 1, half of the copper rockfish (n=10) from the 2.0 DO treatment were removed after completing escape trials due to fish developing symptoms of gas bubble disease (e.g. individuals exhibiting subcutaneous gas embolisms, disoriented swimming), presumably caused by supersaturation of nitrogen in the seawater. Thus, in the first year, there was no replicate tank for the 2.0 DO treatment for brain lateralization or any of the physiology trials. Consequently, only half the number of intended brain lateralization and physiology trials were conducted for 2.0 DO treatment fish. Most (9/10) of the symptomatic individuals recovered, but were not reintroduced to treatment.

Serious fish health issues also started to emerge in Year 2 prior to respirometry trials; both copper and black rockfish across all treatments began to show signs of what was subsequently diagnosed as vitamin C deficiency at the onset of standard metabolic rate (SMR) trials. The main symptoms included growth reductions and spinal cord deformities. Fish were being fed ad-lib, but it was later discovered that a diet comprised solely of krill is deficient in vitamin C for long-term health, and symptoms can take up to 6-months to manifest (personal communication with aquarist staff at Monterey Bay Aquarium). While only healthy individuals (i.e. those that did not display symptoms of vitamin C deficiency) were used in respirometry trials, it is possible that pH and DO effects on fish respirometry during Year 2 were confounded by declining fish health, even if fish were not yet showing symptoms. No results for fish from the reduced DO treatment (4.0 DO) are reported for respirometry trials due to a high number of fish within this treatment developing serious health issues.

Year 1: Independent pH & DO Effects

Behavioral trials

Escape response. Across all treatments, individual variability in escape time was extremely high, and no significant effect of reduced DO or pH on escape time for copper rockfish was detected (Figure 8, Tables 3-4). However, there was a trend of shorter escape times in response to reduced DO, especially in the 2.0 DO treatment (although not statistically significant at $\alpha = 0.05$, $P = 0.059$). Mean escape time in the 6.0, 4.0, and 2.0 DO treatments decreased by 38%, 49%, and 53% respectively compared to the control group. Mean escape times for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials* section.

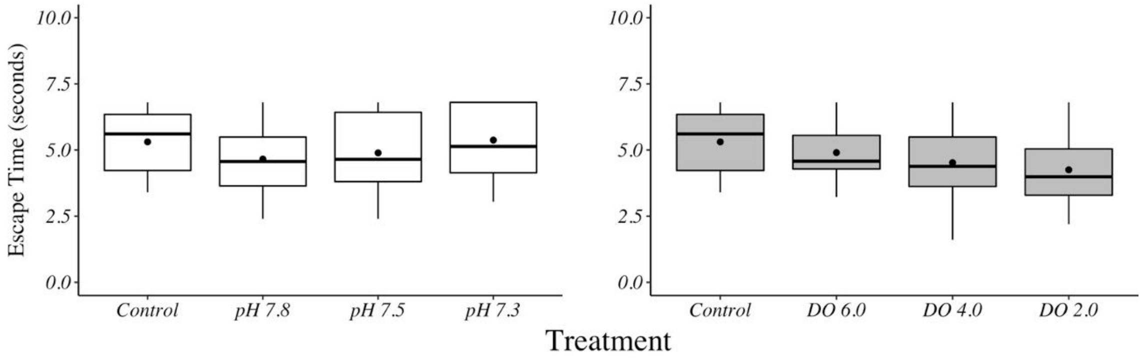

Figure 8. Left panel: Effect of reduced pH on escape time (log-transformed) in juvenile copper rockfish (n=19-20 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on escape time was detected in the linear mixed effect model (P > 0.05). Right panel: Effect of reduced DO on escape time (log-transformed) (n=19-20 fish/treatment) under ambient pH (~7.95). No significant effect of DO on escape time was detected in the linear mixed effect model (P > 0.05). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile.

Table 3. Linear mixed model results for the fixed effects of reduced DO and length on escape time (log-transformed) in juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t - value*	*Pr (>|t|)*
DO 6.0	-0.451	0.411	4	-1.098	0.334
DO 4.0	-0.814	0.410	4	-1.985	0.118
DO 2.0	-1.064	0.407	4	-2.616	0.059
Length	0.013	0.026	69	0.502	0.617

Table 4. Linear mixed model results for the fixed effects of reduced pH and length on escape time (log-transformed) in juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t - value*	*Pr (>|t|)*
pH 7.8	-0.871	0.427	4	-2.042	0.111
pH 7.5	-0.594	0.418	4	-1.421	0.228
pH 7.3	-0.097	0.416	4	-0.233	0.827
Length	0.039	0.025	71	1.567	0.122

<u>Relative brain lateralization.</u> Variability in relative brain lateralization amongst individuals was high in all treatments. There was no significant effect of reduced DO or pH on relative brain lateralization index (L_R) in copper rockfish (Figure 9, Tables 5-6). Mean relative lateralization indices for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

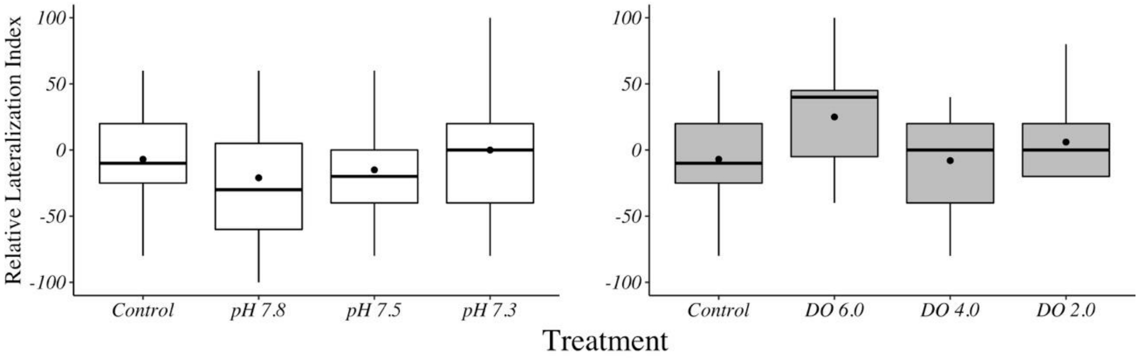

Figure 9. Left panel: Independent effect of reduced pH on relative brain lateralization (L_R) of juvenile copper rockfish (n=10-20 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on L_R was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent effect of reduced DO on L_R (n=20 fish/treatment) under ambient pH (~7.95). No significant effect of DO on L_R was detected in linear mixed effect model ($P > 0.05$). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile.

Table 5. Linear mixed effect model results for the fixed effects of reduced DO and length on relative brain lateralization (L_R) of juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	34.878	11.909	3	2.929	0.061
DO 4.0	-0.424	11.816	3	-0.036	0.974
DO 2.0	9.115	14.611	3	0.624	0.577
Length	-1.431	0.760	62	-1.892	0.063

Table 6. Linear mixed effect model results for the fixed effects of reduced pH and length on relative brain lateralization (L_R) of juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	-13.964	15.566	4	-0.897	0.420
pH 7.5	-7.952	15.587	4	-0.510	0.637
pH 7.3	7.036	15.566	4	0.452	0.675
Length	-0.029	0.090	71	-0.039	0.969

<u>Absolute brain lateralization.</u> Individual variability in absolute brain lateralization (L_A) was high across all treatments. No significant effect of pH or DO on L_A was detected (Figure 10, Tables 7-8). A significant effect of fish size (total length) on absolute lateralization was observed in fish exposed to various DO concentrations, however, this affect appears to be largely driven by one fish (Figure 11; model was rerun with outlier removed and results did not change). Mean absolute lateralization indices for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

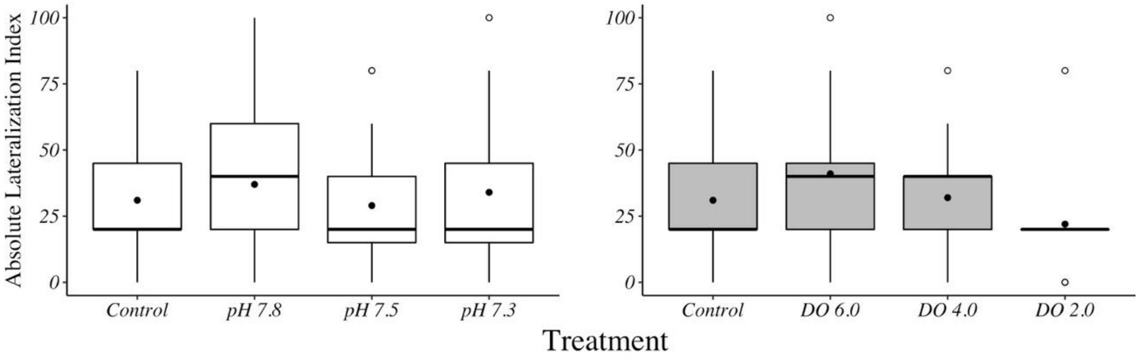

Figure 10. Left panel: Independent effect of reduced pH on absolute brain lateralization (L_A) of juvenile copper rockfish (n=10-20 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on L_A was detected in the linear mixed effect model (P > 0.05). Right panel: Independent effect of reduced DO on L_A (n=20 fish/treatment) under ambient pH (~7.95). No significant effect of DO on L_A was detected in the linear mixed effect model (P > 0.05). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 7. Linear mixed effect model results for the fixed effects of reduced DO and length on absolute brain lateralization (L_A) of juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-values are marked in bold.

Fixed effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	11.753	10.151	3	1.158	0.331
DO 4.0	1.351	10.116	3	0.133	0.902
DO 2.0	-11.367	12.442	3	-0.914	0.428
Length	-0.877	0.431	62	-2.033	**0.046 ***

Table 8. Linear mixed effect model results for the fixed effects of reduced pH and length on absolute brain lateralization (L_A) of juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	5.601	10.597	4	0.529	0.625
pH 7.5	-2.526	10.610	4	-0.238	0.824
pH 7.3	2.601	10.597	4	0.245	0.818
Length	0.319	0.486	71	0.656	0.514

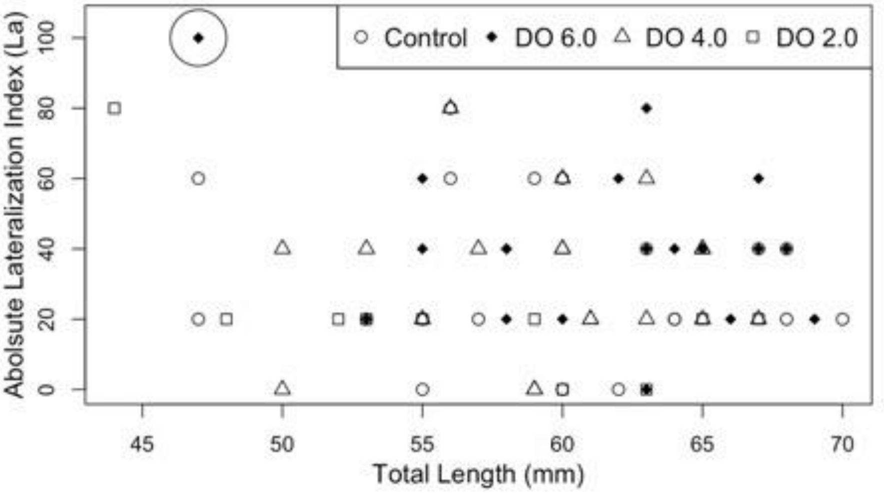

Figure 11. Effect of copper rockfish size (total length) on absolute lateralization index (L_A) across control and reduced DO treatments. Influential data point is encircled.

Physiology trials

Critical swimming speed (U_{crit}). Swimming performance of copper rockfish declined significantly in response to the 4.0 and 2.0 DO conditions (Figure 12, Table 9). Swimming performance exhibited a nearly linear decline over the range of DO conditions examined: suggesting that a drop in DO by ~30% translates approximately to a ~15% decline in swimming performance. Mean U_{crit} scores in the 6.0, 4.0, and 2.0 DO treatments decreased by 15%, 28%, and 40% respectively compared to the control group. Swimming performance also declined in the pH 7.8, pH 7.5, and pH 7.3 treatments, but these effects did not differ significantly from control (Figure 12, Table 10). Mean U_{crit} scores in the pH 7.8, 7.5, and 7.3 treatments decreased by 18%, 15%, and 20% respectively compared to the control group. These results suggest a consistent, step-wise drop in U_{crit} in response to various pH levels, rather than a linear decline in U_{crit} as demonstrated in response to DO. Variability in swimming performance was greatest amongst individuals within the control group. Mean U_{crit} for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

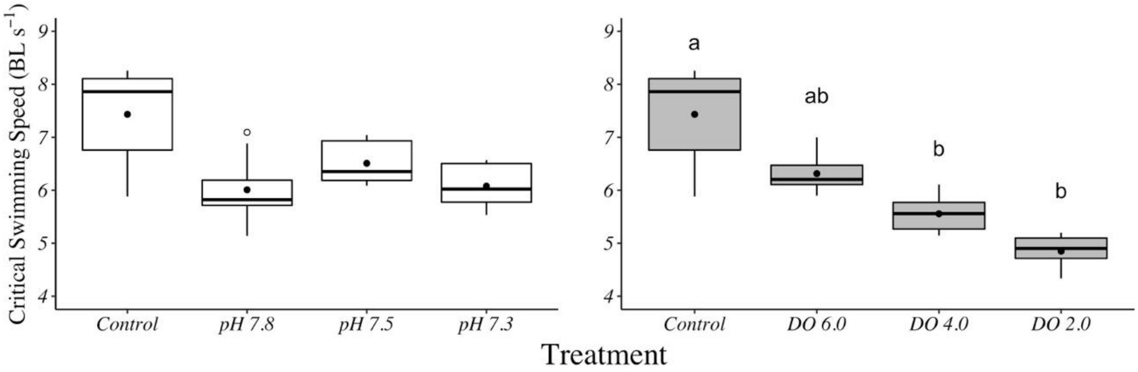

Figure 12. Left panel: Independent effect of reduced pH on critical swimming speed (U_{crit}) of juvenile copper rockfish (n=6-8 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on U_{crit} was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent effect of reduced DO on U_{crit} (n=8-10 fish/treatment) under ambient pH (~7.95). Letters indicate Tukey post-hoc pairwise comparisons between control and reduced DO treatment means. U_{crit} for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 9. Linear mixed effect model results for the fixed effects of reduced DO and length on critical swimming speed (U_{crit}) of juvenile copper rockfish. U_{crit} for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-values are marked in bold.

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	-1.204	0.595	3	-2.026	0.136
DO 4.0	-2.137	0.594	3	-3.594	**0.037 ***
DO 2.0	-2.785	0.707	3	-3.938	**0.029 ***
Length	0.050	0.040	23	1.243	0.226

Table 10. Linear mixed effect model results for the fixed effects of reduced pH and length on critical swimming speed (U_{crit}) of juvenile copper rockfish. U_{crit} for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	-1.407	0.563	4	-2.499	0.067
pH 7.5	-0.999	0.570	4	-1.755	0.154
pH 7.3	-1.398	0.566	4	-2.469	0.069
Length	0.051	0.029	26	1.741	0.094

Standard metabolic rate (SMR). Dissolved oxygen concentration remained relatively stable and near the desired set points throughout SMR trials (Figures 14-15). Temperature during SMR trials varied between ~10.5 - 12.5 ° (Figures 16-17). pH conditions were spot checked prior to and after SMR trials, but were not continuously monitored. Standard metabolic rate (SMR), corrected for temperature, varied widely among individual copper rockfish. While SMR was also not significantly affected by reduced DO, mean SMR decreased in the 6.0, 4.0, and 2.0 DO treatments by 22%, 20%, and 22% respectively (Figure 13, Table 11). Reduced pH did not significantly affect SMR (Figure 13, Table 12). Mean SMR for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

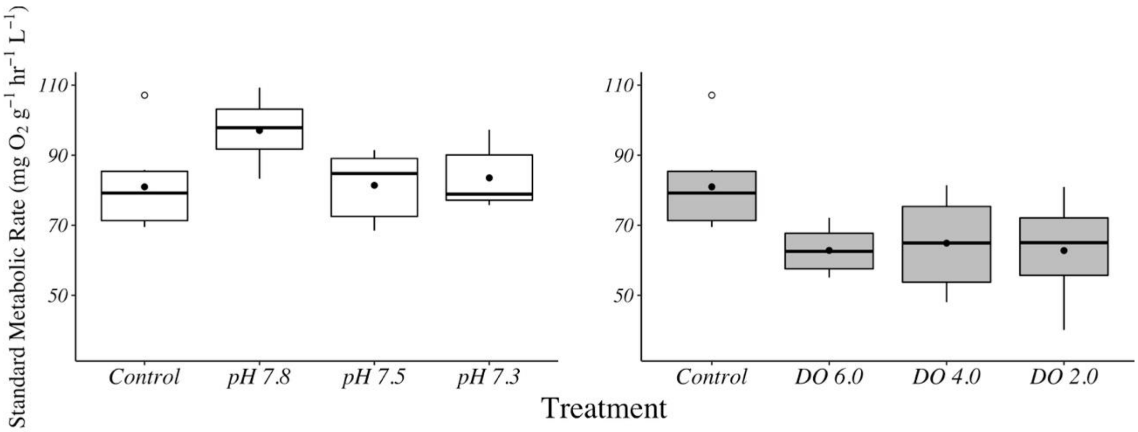

Figure 13. Left panel: Independent effect of reduced pH on standard metabolic rate (SMR) of juvenile copper rockfish (n=5-8 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on SMR was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent effect of reduced DO on SMR (n=4-8 fish/treatment) under ambient pH (~7.95). No significant effect of DO on SMR was detected in the linear mixed effect model ($P > 0.05$). SMR for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship**.** The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile.

Table 11. Linear mixed effect model results for the fixed effects of reduced DO and weight on standard metabolic rate (SMR) of juvenile copper rockfish. SMR for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	-19.960	7.807	3	-2.557	0.083
DO 4.0	-19.114	7.749	3	-2.467	0.090
DO 2.0	-20.706	9.588	3	-2.159	0.120
Weight	-1.018	0.799	22	-1.274	0.216

Table 12. Linear mixed effect model results for the fixed effects of reduced pH and weight on standard metabolic rate (SMR) of juvenile copper rockfish. SMR for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
PH 7.8	18.417	11.111	4	1.658	0.173
PH 7.5	3.265	10.917	4	0.299	0.780
PH 7.3	0.179	14.652	4	0.016	0.988
Weight	-0.823	0.998	18	-0.825	0.420

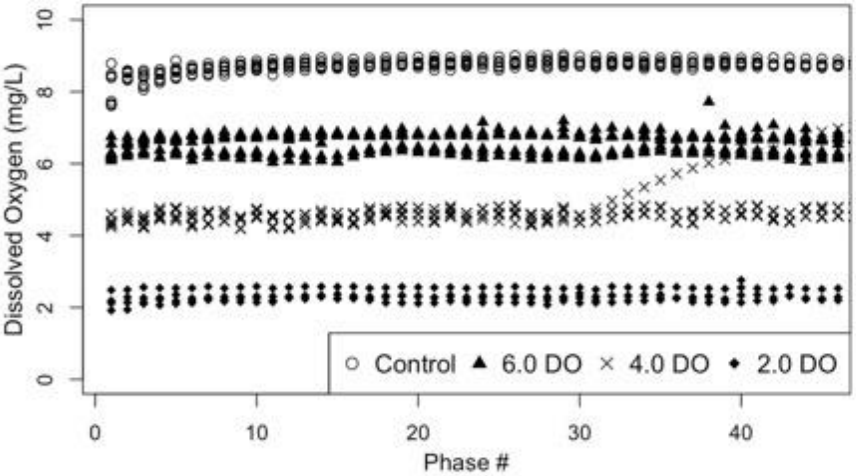

Figure 14. Average dissolved oxygen (DO) concentration for each phase (measurement period) recorded during Year 1 SMR trials for copper rockfish exposed to various DO treatments. Each record applies to up to 4 fish being tested simultaneously.

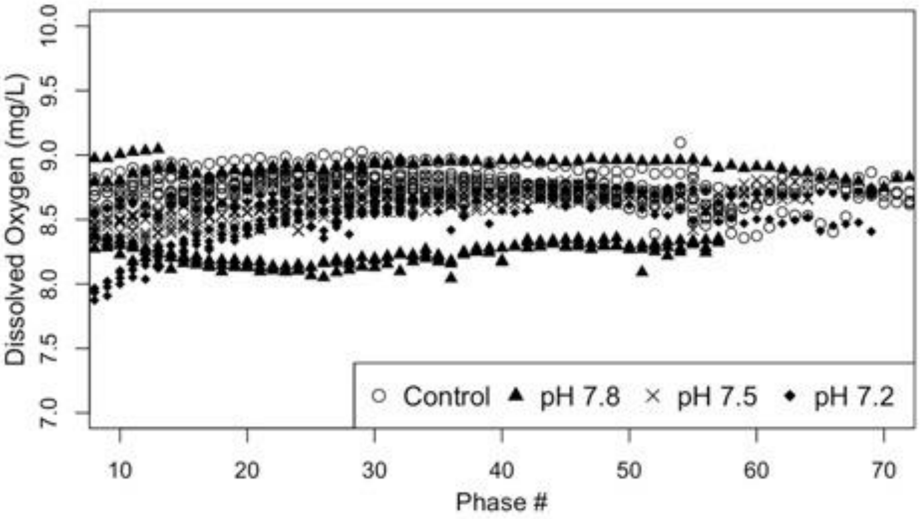

Figure 15. Average dissolved oxygen concentrations (DO) for each phase (measurement period) recorded during Year 1 SMR trials for copper rockfish exposed to various pH treatments. Each record applies to up to 4 fish being tested simultaneously.

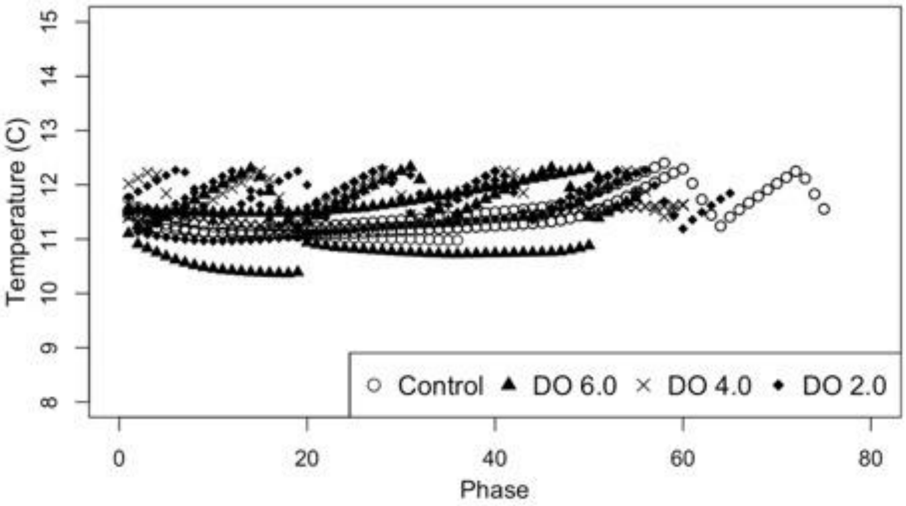

Figure 16. Average temperature (°C) for each phase (measurement period) recorded during Year 1 SMR trials for copper rockfish exposed to various DO treatments. Each record applies to up to 4 fish being tested simultaneously.

Figure 17. Average temperature (C) for each phase (measurement period) recorded during Year 1 SMR trials for copper rockfish exposed to various DO treatments. Each record applies to up to 4 fish being tested simultaneously

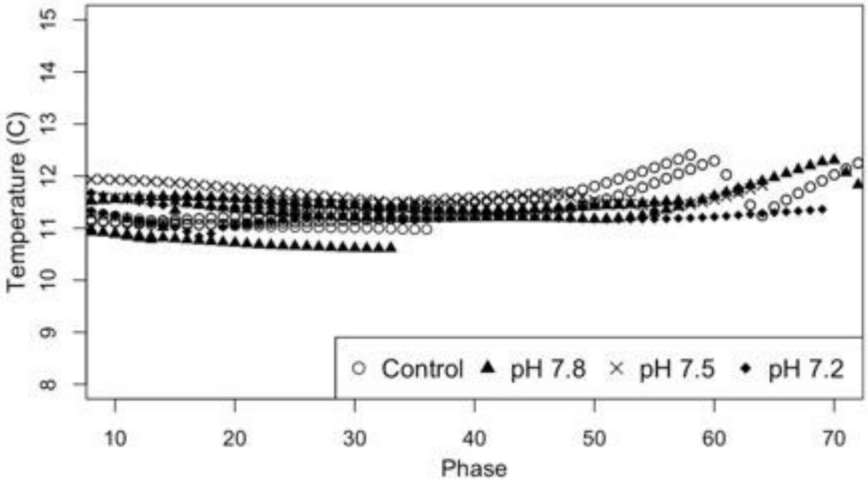

Figure 18. Average temperature (°C) for each phase (measurement period) recorded during Year 1 SMR trials for copper rockfish exposed to various pH treatments. Each record applies to up to 4 fish being tested simultaneously.

Maximum metabolic rate (MMR). Individual variability in maximum metabolic rate (MMR) was high across all treatments for copper rockfish, and no significant effect of pH or DO on MMR was detected (Figure 19, Tables 13-14). However, the pattern of responses to MMR suggest the possibility that MMR might be reduced at very low DO (~2.0 mg/L). Mean MMR for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

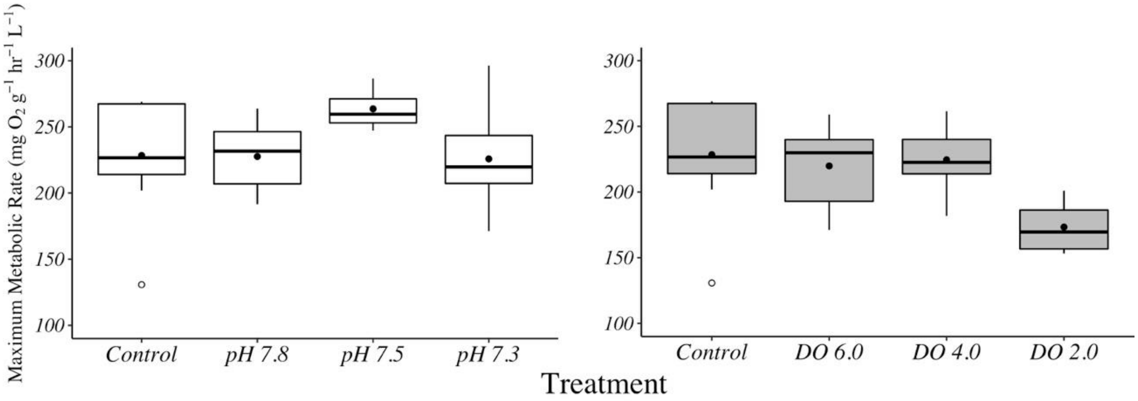

Figure 19. Left panel: Independent effect of reduced pH on maximum metabolic rate (MMR) of juvenile copper rockfish (n=4-8 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on MMR was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent effect of reduced DO on MMR (n=8-9 fish/treatment) under ambient pH (~7.95). No significant effect of pH on MMR was detected in the linear mixed effect model ($P > 0.05$). MMR for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 13. Linear mixed effect model results for the fixed effects of reduced DO and weight on maximum metabolic rate (MMR) of copper rockfish. MMR for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	-7.437	18.323	5	-0.406	0.702
DO 4.0	-2.248	28.205	5	-0.124	0.907
DO 2.0	-53.781	22.698	5	-2.369	0.064
Weight	-0.901	2.651	19	-0.340	0.738

Table 14. Linear mixed effect model results for the fixed effects of reduced pH and weight on maximum metabolic rate (MMR) of copper rockfish. MMR for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	2.278	18.572	9	-0.123	0.905
pH 7.5	36.325	17.448	9	2.082	0.067
pH 7.2	-1.215	17.043	9	-0.071	0.945
Weight	1.446	3.186	20	0.454	0.655

Aerobic scope. Aerobic scope of copper rockfish demonstrated highly individual variability and sample sizes were small. There was no significant effect of pH on aerobic scope of copper rockfish, and results demonstrated counter-intuitive and non-monotonic patterns (Figure 20, Tables 15-16). However, aerobic scope did demonstrate a 28% decline in response to the 2.0 DO treatment, but the effect was not statistically significant at $\alpha = 0.05$ ($P = 0.069$). Mean aerobic scope for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

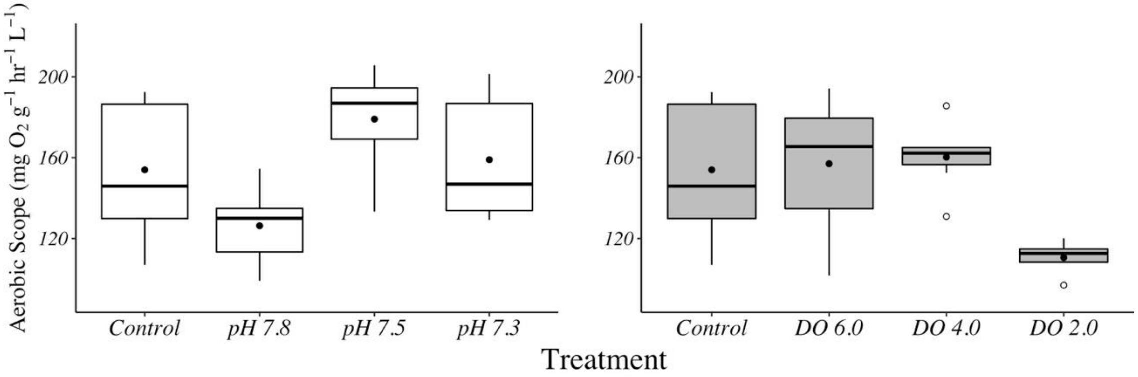

Figure 20. Left panel: Independent effect of reduced pH on aerobic scope of juvenile copper rockfish (n=4-8 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on MMR was detected in the linear mixed effect model (P > 0.05). Right panel: Independent effect of reduced DO on aerobic scope (n=5-7 fish/treatment) under ambient pH (~7.95). No significant effect of DO on MMR was detected in the linear mixed effect model (P > 0.05). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 15. Linear mixed effect model results for the fixed effects of reduced DO on aerobic scope of juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	3.008	14.580	4	0.206	0.847
DO 4.0	6.490	15.079	4	0.430	0.689
DO 2.0	-43.616	17.667	4	-2.469	0.069
Weight	0.650	2.395	17	0.271	0.789

Table 16. Linear mixed effect model results for the fixed effects of reduced pH on aerobic scope of juvenile copper rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	-27.797	17.581	9	-1.581	0.148
pH 7.5	24.991	16.189	9	1.544	0.157
pH 7.3	4.965	16.591	9	0.299	0.772
Weight	-0.055	2.853	12	-0.019	0.985

Critical oxygen level (P_{crit}). Critical oxygen level (P_{crit}) was not significantly affected by reduced pH or DO (Figure 21, Tables 17-18), but trends suggest a possible decline in response to both stressors. An effect of fish size (weight) on P_{crit} was observed, reflecting a tendency for larger fish to exhibit higher P_{crit} than smaller fish (Figures 22-23). However, the pattern is not consistently observed across all treatments. Mean P_{crit} for rockfish exposed to various pH and DO levels are summarized in the *Year 1: Comparisons Across Experimental Trials section*.

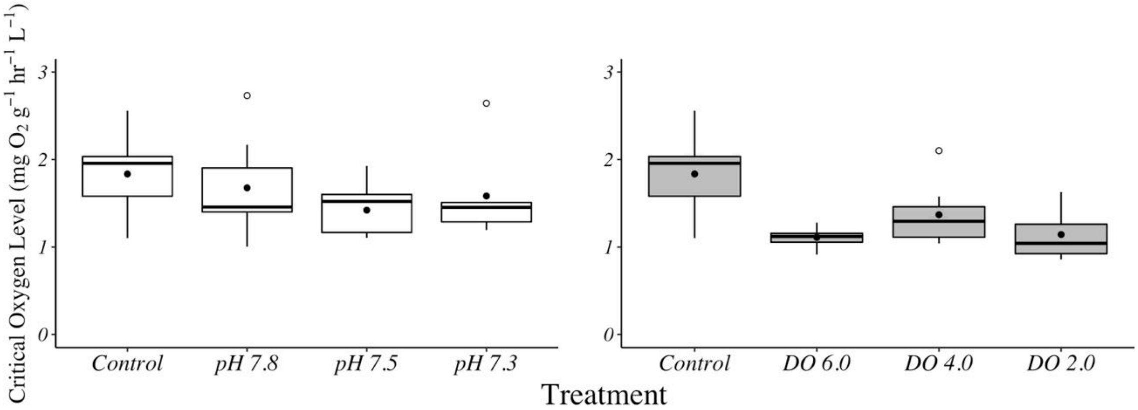

Figure 21. Left panel: Independent effect of reduced pH on critical oxygen level (P_{crit}) of juvenile copper rockfish (n=4-8 fish/treatment) under ambient DO (~8.5 mg/L). No significant effect of pH on P_{crit} was detected in the linear mixed effect model (P > 0.05). Right panel: Independent effect of reduced DO on P_{crit} (n=7-9 fish/treatment) under ambient pH (~7.95). No significant effect of DO on P_{crit} was detected in the linear mixed effect model (P > 0.05). P_{crit} for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 17. Linear mixed effect model results for the fixed effects of reduced DO and weight on critical oxygen level (P_{crit}) of juvenile copper rockfish. P_{crit} for copper rockfish was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-values are marked in bold.

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	-0.685	0.346	2	-1.977	0.187
DO 4.0	-0.324	0.351	2	-0.925	0.453
DO 2.0	-0.705	0.403	2	-1.749	0.223
Weight	0.070	0.023	19	2.932	**0.009 ***

Table 18. Linear mixed effect model results for the fixed effects of reduced DO and weight on critical oxygen level (P_{crit}) of juvenile copper rockfish. P_{crit} for copper rockfish was scaled to a standard temperature (12 °C) using a Q10 (=2) relationship**.** The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-values are marked in bold.

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	0.070	0.465	4	0.152	0.887
pH 7.5	-0.300	0.450	4	-0.666	0.542
pH 7.3	0.139	0.462	4	0.301	0.779
Weight	0.075	0.034	24	2.214	**0.037 ***

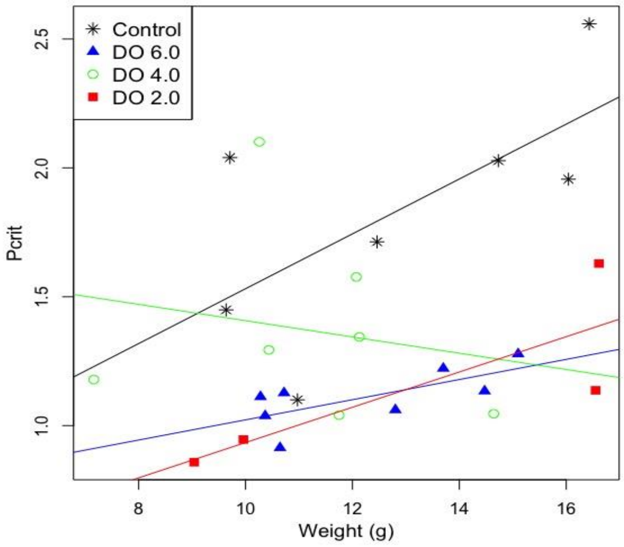

Figure 22. Effect of copper rockfish size (wet weight) on critical oxygen level (P_{crit}) across control and reduced DO treatments. Fitted regression lines of P_{crit} as a function of fish size for each treatment are displayed in color. Each point represents an individual fish.

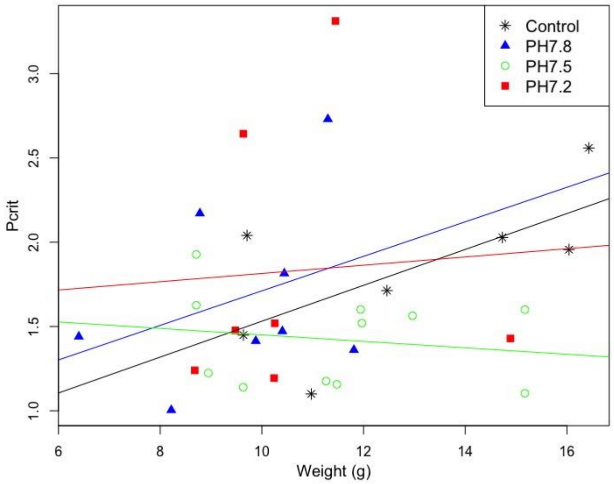

Figure 23. Effect of copper rockfish size (wet weight) on critical oxygen level (P_{crit}) across control and reduced pH treatments. Fitted regression lines of P_{crit} as a function of fish size for each treatment are displayed in color. Each point represents an individual fish.

Somatic growth. Somatic growth rate (mm/day & g/day), which was analyzed under two different time intervals (after 57-69 and 223-276 days of exposure to treatment), was not significantly affected by reduced pH or DO (Figures 24-25, Tables 19-24). However, there is a suggestive trend of reduced somatic growth (both in terms of length and weight) after 57-69 days of exposure to the 2.0 DO treatment. Model results for the effect of 223-276 days of exposure to treatment on fish weight (g/day) are not included in results, but also proved to be not significant.

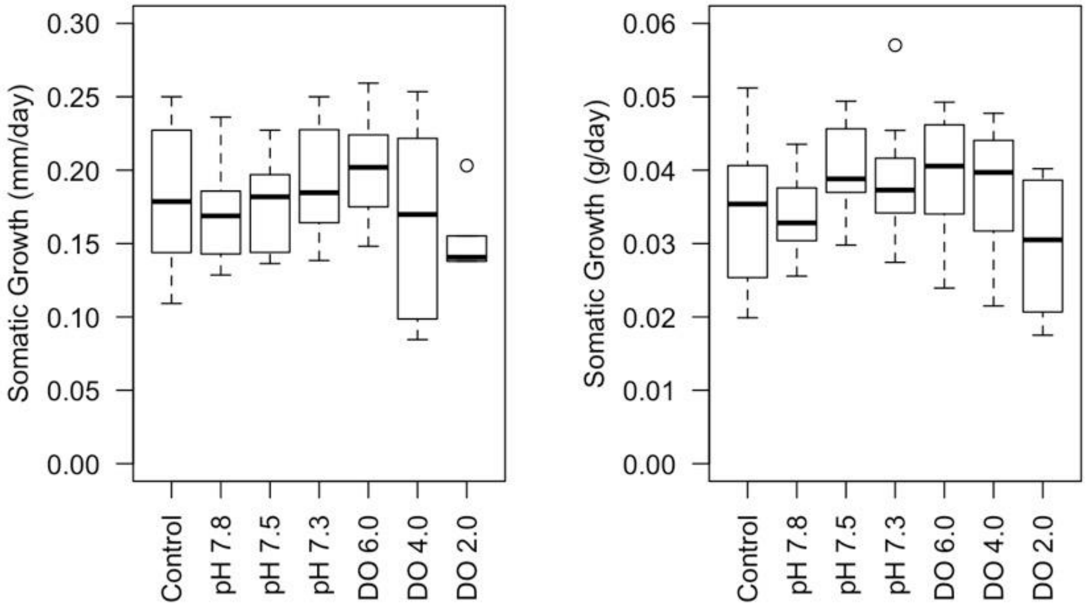

Figure 24. Left panel: Somatic growth rate (mm/day) for a subset of juvenile copper rockfish (n=6-10 fish/treatment), measured prior to treatment initiation and prior to U_{crit} trials (57-69 days in treatment). No significant effect of pH or DO on somatic growth rate (mm/day) was detected in the linear mixed effect models (P > 0.05). Right panel: Somatic growth rate (g/day) for a subset of juvenile copper rockfish (n=6-10 fish/treatment), measured prior to treatment initiation and prior to U_{crit} trials (57-69 days in treatment). No significant effect of pH or DO on somatic growth rate (g/day) was detected in the linear mixed effect models (P > 0.05). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

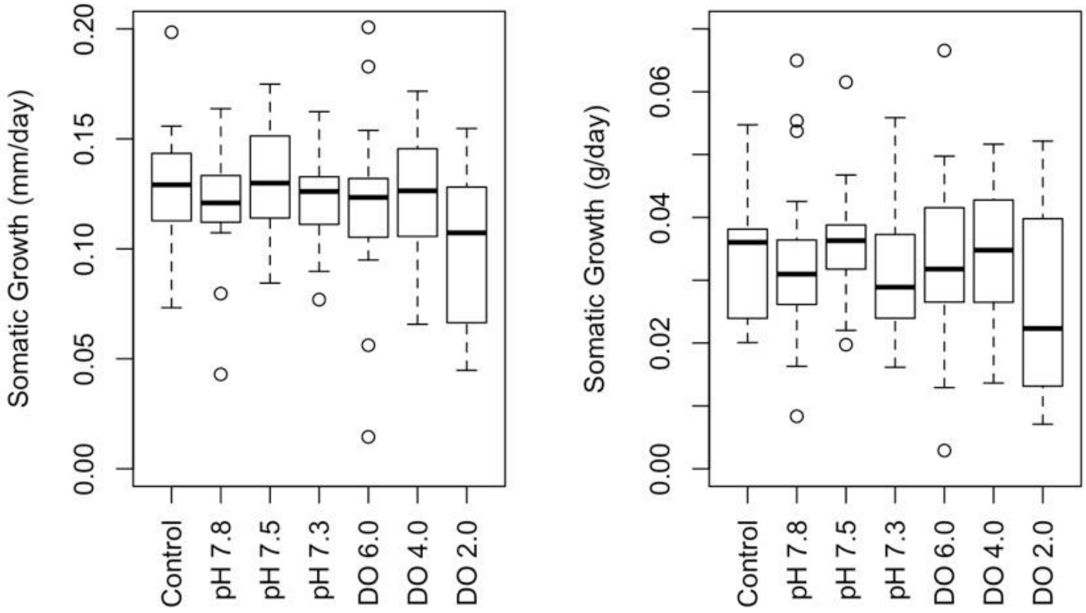

Figure 25. Left panel: Somatic growth rate (mm/day) for all juvenile copper rockfish that survived from treatment initiation until the end of the experiment (n=8-20 fish/treatment), measured prior to escape trials and prior to fish dissections (223-276 days in treatment). No significant effect of pH or DO on somatic growth rate (mm/day) was detected in the linear mixed effect models ($P > 0.05$). Right panel: Somatic growth rate (g/day) for all juvenile copper rockfish that survived from treatment initiation until the end of the experiment (n=8-20 fish/treatment), measured prior to escape trials and prior to fish dissections (223-276 days in treatment). No significant effect of pH or DO on somatic growth rate (g/day) was detected in the linear mixed effect models ($P > 0.05$). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 19. Linear mixed effect model results for the fixed effect of reduced DO on somatic growth rate (mm/day) of juvenile copper rockfish during the first 57-69 days in treatment. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	0.018	0.026	4	0.691	0.527
DO 4.0	-0.019	0.027	4	-0.687	0.530
DO 2.0	-0.030	0.028	4	-1.064	0.347

Table 20. Linear mixed effect model results for the fixed effect of reduced DO on somatic growth rate (g/day) of juvenile copper rockfish during the first 57-69 days in treatment. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	0.004	0.007	4	0.584	0.591
DO 4.0	0.003	0.007	4	0.394	0.714
DO 2.0	-0.006	0.005	4	-0.806	0.465

Table 21. Linear mixed effect model results for the fixed effect of reduced pH on somatic fish growth (mm/day) of juvenile copper rockfish during the first 57-69 days in treatment. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	-0.010	0.021	4	-0.483	0.654
pH 7.5	-0.008	0.023	4	-0.330	0.758
pH 7.3	0.011	0.023	4	0.481	0.655

Table 22. Linear mixed effect model results for the fixed effect of reduced pH on somatic fish growth (g/day) of juvenile copper rockfish during the first 57-69 days in treatment. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	-0.006	0.005	4	-0.135	0.899
pH 7.5	0.006	0.005	4	1.266	0.274
pH 7.3	0.004	0.005	4	0.959	0.392

Table 23. Linear mixed effect model results for the fixed effect of reduced DO on somatic growth rate (mm/day) of juvenile copper rockfish after 223-276 days in treatment. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
DO 6.0	-0.0009	0.004	3	-0.207	0.849
DO 4.0	0.0002	0.005	3	0.056	0.959
DO 2.0	-0.0080	0.006	3	-1.384	0.260

Table 24. Linear mixed effect model results for the fixed effect of reduced pH on somatic growth rate (mm/day) of juvenile copper rockfish after 223-276 days in treatment. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Fixed Effects	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
pH 7.8	-0.0008	0.004	4	-0.192	0.857
pH 7.5	0.0013	0.004	4	0.334	0.755
pH 7.3	-0.0025	0.003	4	-0.638	0.558

Year 1: Comparisons across experimental trials. Behavioral responses (escape time, relative lateralization, absolute lateralization) and physiological responses (swimming performance (U_{crit}), standard metabolic rate (SMR), maximum, metabolic rate (MMR), aerobic scope, critical oxygen level (P_{crit}), and somatic growth (mm/day) during the first 57-69 days in treatment) for copper rockfish across all treatments are summarized in Table 25 & Table 26. Overall, copper rockfish exhibited greater changes in physiological performances in response to reduced DO than reduced pH. Behavioral responses were variable across all treatments and not significantly affected by either stressor. Fish from the control treatment exhibited the highest swimming performance and highest critical oxygen level (P_{crit}). Fish from the 2.0 DO treatment exhibited the worst swimming performance, lowest SMR, lowest MMR, and lowest aerobic scope.

Table 25. Mean escape time (seconds), relative brain lateralization (L_R), absolute brain lateralization (La), associated standard error and sample size (N) for all copper rockfish exposed to Year 1 treatment conditions.

Treatment	*Escape Time (s)*	*Relative Lateralization (L_R)*	*Absolute Lateralization (L_A)*
Control	371.4 +/- 77.9 N = 20	-7 +/- 8.62 N = 20	31 +/- 5.1 N = 20
DO 6.0	229.7 +/- 59.6 N = 20	25 +/- 9.1 N = 20	41 +/- 5.1 N = 20
DO 4.0	189.1 +/- 53.5 N = 19	-8 +/- 8.5 N = 20	32 +/- 4.7 N = 20
DO 2.0	173.8 +/- 61.7 N = 19	6 +/- 9.9 N = 10	22 +/- 7.0 N = 10
pH 7.8	203.1 +/- 58.4 N = 20	-21 +/- 9.2 N = 10	37 +/- 6.0 N = 20
pH 7.5	298.8 +/- 79.6 N = 20	-15 +/- 8.1 N = 20	29 +/- 5.7 N = 20
pH 7.3	425.3 +/- 90.4 N = 20	0 +/- 10.5 N = 20	34 +/- 7.0 N = 20

Table 26. Mean swimming performance (U_{crit}), standard metabolic rate (SMR), maximum, metabolic rate (MMR), aerobic scope, critical oxygen level (P_{crit}), and somatic growth (mm/day during the first 57-69 days in treatment), associated standard errors and sample size (N) across all copper rockfish exposed to Year 1 treatment conditions.

Treatment	U_{crit}	*SMR*	*MMR*	*Aerobic Scope*	P_{crit}	*Somatic growth*
Control	7.43 +/- 0.32 N = 8	80.97 +/- 4.45 N = 8	228.44 +/- 15.06 N = 9	153.10 +/- 13.22 N = 7	1.84 +/- 0.18 N = 7	0.18 +/- 0.02 N = 7
DO 6.0	6.31 +/- .11 N = 9	62.82 +/- 2.29 N = 8	219.86 +/- 10.90 N = 8	157.04 +/- 11.56 N = 8	1.11 +/- 0.04 N = 8	0.17 +/- 0.01 N = 10
DO 4.0	5.34 +/- .24 N = 8	64.91 +/- 3.86 N = 10	224.57 +/- 8.56 N = 8	160.27 +/- 6.20 N = 7	1.37 +/- 0.14 N = 7	0.18 +/- 0.01 N = 7
DO 2.0	4.58 +/- 0.30 N = 6	62.78 +/- 8.69 N = 4	173.33 +/- 11.11 N = 4	110.55 +/- 4.88 N = 4	1.14 +/- 0.17 N = 4	0.19 +/- 0.01 N = 7
pH 7.8	6.01 +/- .19 N = 10	102.74 +/- 5.02 N = 6	227.62 +/- 9.37 N = 8	126.32 +/- 7.54 N = 6	1.68 +/- 0.19 N = 8	0.20 +/- 0.01 N = 8
pH 7.5	6.51 +/- .15 N = 8	86.05 +/- 5.82 N = 7	263.65 +/- 5.21 N = 8	179.05 +/- 9.29 N = 7	1.42 +/- 0.08 N = 11	0.16 +/- 0.03 N = 7
pH 7.3	6.08 +/- .13 N = 9	83.54 +/- 3.83 N = 6	225.82 +/- 14.01 N = 9	159.00 +/- 13.44 N = 6	1.83 +/- 0.31 N = 7	0.15 +/- 0.01 N = 6

Year 2: Species-Specific Independent and Combined pH & DO Effects

Behavioral trials

Escape response. Individual variability in escape response was extremely high in escape response of black rockfish, ranging from nearly instantaneous to effectively zero (i.e., no escape within 30 minutes). Overall, copper rockfish demonstrated faster and more consistent escape responses compared to black rockfish. Changes in individuals' escape times for copper and black rockfish were not significantly affected by independent or simultaneous exposure to reduced pH and DO (Figure 26, Table 27). Mean changes in escape time for copper and black rockfish exposed to reduced pH and DO are summarized in the *Year 2: Comparisons Across Experimental Trials* section.

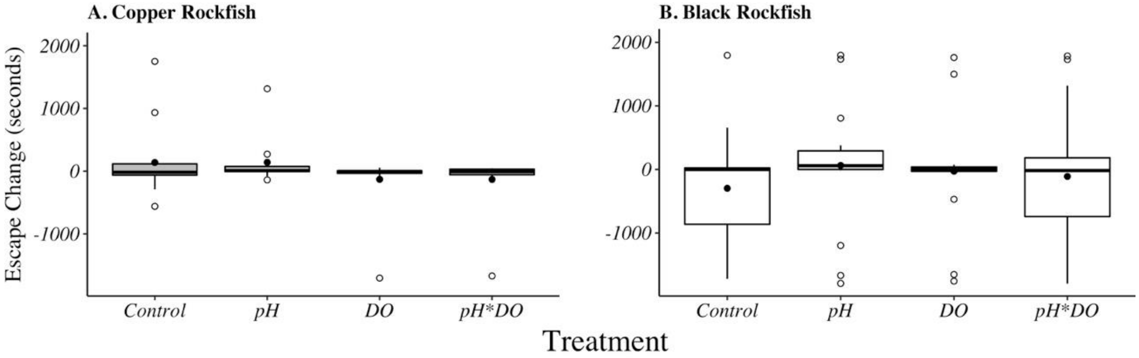

Figure 26. Left panel: Independent and combined effect of reduced pH and DO on changes in escape time (seconds) for juvenile copper rockfish (n=11-15 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on copper rockfish escape response was detected in the linear mixed effect model (P > 0.05). Right panel: Independent and combined effect of reduced pH and DO on changes in escape time (seconds) for juvenile black rockfish (n=17-19 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on black rockfish escape response was detected in the linear mixed effect model (P > 0.05). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 27. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (length) on changes in escape time (seconds) for juvenile copper and black rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	-10.698	230.956	4	-0.046	0.963
	DO 4.0	-272.124	220.694	4	-1.233	0.285
	pH 7.5 * DO 4.0	-298.838	224.643	4	-1.330	0.254
	Length	5.609	18.879	43	0.297	0.768
Black	pH 7.5	380.845	308.227	4	1.236	0.284
	DO 4.0	263.561	316.488	4	0.833	0.452
	pH 7.5 * DO 4.0	-415.039	441.934	4	-0.939	0.401
	Length	21.238	16.724	63	1.270	0.209

Relative brain lateralization. Individual variability in relative brain lateralization (L_R) was high across all treatments for both species. L_R in copper rockfish was significantly affected by exposure to reduced DO, suggesting a shift from left to right in turning preference (Figure 27, Table 28). A significant interaction between pH and DO on copper rockfish relative lateralization (L_R) was also detected (Table 28). This interaction was not consistent with a synergistic effect, but rather was consistent with an antagonistic effect, as fish exposed simultaneously to both stressors exhibited a reduced preference for turning right relative to those exposed to only one stressor (Figure 27). There was no independent effect of reduced pH on L_R in copper rockfish. There was no significant effect of pH, DO, or simultaneous exposure to both stressors on L_R in black rockfish (Figure 27, Table 28). Mean relative brain lateralization (L_R) for copper and black rockfish exposed to reduced pH and DO are summarized in the *Year 2: Comparisons Across Experimental Trials* section.

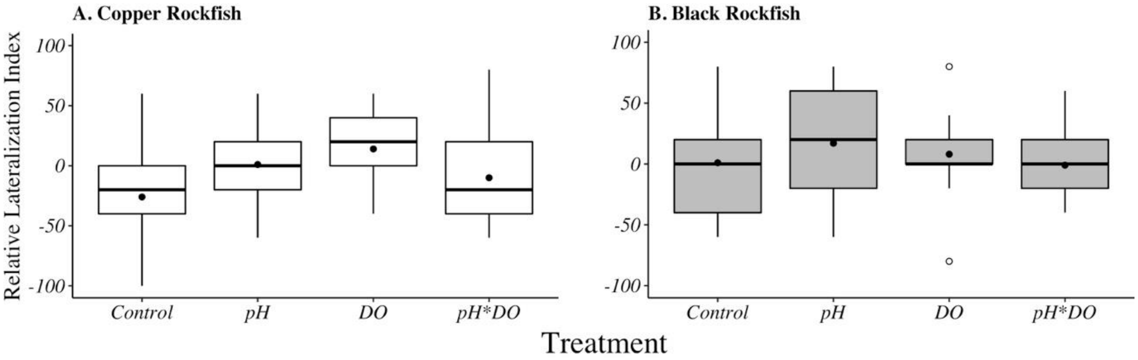

Figure 27. Left panel: Independent and combined effect of reduced pH and DO on relative brain lateralization (L_R) of juvenile copper rockfish (n=20 fish/treatment). Letters indicate Tukey post-hoc pairwise comparisons between copper rockfish treatment means. Right panel: Independent and combined effect of reduced pH and DO on relative brain lateralization (L_R) of juvenile black rockfish (n=20 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on black rockfish L_R was detected in the linear mixed effect model ($P > 0.05$). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 28. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (length) on relative brain lateralization of juvenile copper and black rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	29.150	11.690	4	2.494	0.067
	DO 4.0	41.421	11.415	4	3.629	**0.022 ***
	pH 7.5 * DO 4.0	-54.644	1.139	4	-3.247	**0.032 ***
	Length	0.729	16.828	71	0.640	0.524
Black	pH 7.5	10.990	11.174	4	0.984	0.381
	DO 4.0	14.038	11.192	4	1.254	0.278
	pH 7.5 * DO 4.0	-28.968	15.810	4	-1.832	0.141
	Length	0.040	0.702	71	0.056	0.955

Absolute brain lateralization. Individual variability in absolute brain lateralization (L_A) was high across all treatments for both species. Neither species demonstrated a significant change in absolute lateralization in response to pH, DO, or simultaneous exposure to pH and DO (Figure 28, Table 29).

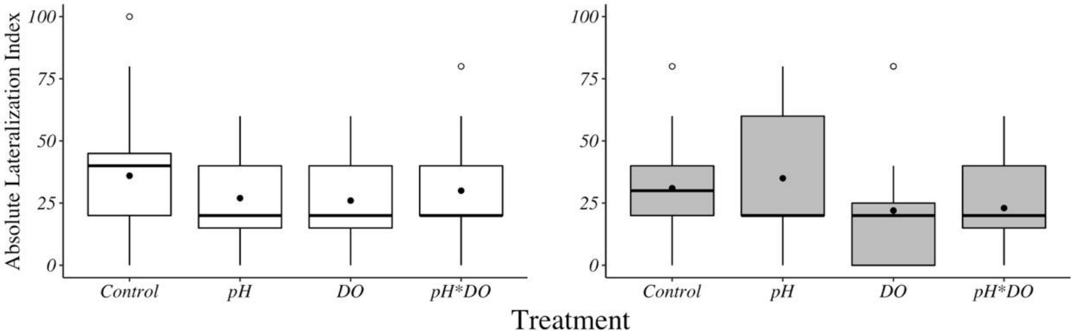

Figure 28. Left panel: Independent and combined effect of reduced pH and DO on absolute brain lateralization (L_A) of juvenile copper rockfish (n=20 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on copper rockfish L_A was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent and combined effect of reduced pH and DO on absolute brain lateralization (L_A) of juvenile black rockfish (n=20 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on black rockfish L_A was detected in the linear mixed effect model ($P > 0.05$). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 29. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (length) on absolute brain lateralization of juvenile copper and black rockfish. Estimates represent the estimated offset from the control and the associated standard error. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	-7.028	9.022	4	-0.779	0.480
	DO 4.0	-8.696	8.868	4	-0.981	0.382
	pH 7.5 * DO 4.0	9.657	12.926	4	0.747	0.497
	Length	0.669	0.750	71	0.891	0.376
Black	pH 7.5	0.950	7.149	4	0.132	0.901
	DO 4.0	-5.811	7.161	4	-0.811	0.463
	pH 7.5 * DO 4.0	-2.841	10.115	4	-0.281	0.793
	Length	0.199	10.115	71	0.443	0.659

Physiology trials

Critical swimming speed (U_{crit}). Critical swimming speed (U_{crit}) of copper rockfish declined significantly under reduced DO conditions, and also declined (non-significantly) under reduced pH (P = 0.057) and under simultaneous exposure to both stressors (P = 0.206) (Figure 29, Table 30). Swimming performance in copper rockfish decreased in the reduced pH, reduced DO, and combined treatment by 11%, 16%, and 19% respectively compared to control. Similarly, U_{crit} of black rockfish also declined following exposure to reduced DO (but the effect was not significant at $\alpha = 0.05$; P = 0.056), reduced pH, and simultaneous exposure to both stressors (Figure 29, Table 30).

U_{crit} in black rockfish declined in the reduced pH, reduced DO, and combined treatment by 12%, 18%, and 20% respectively compared to control. Mean U_{crit} scores of copper and black rockfish across all treatments are summarized in the *Year 2: Comparisons Across Experimental Trials* section.

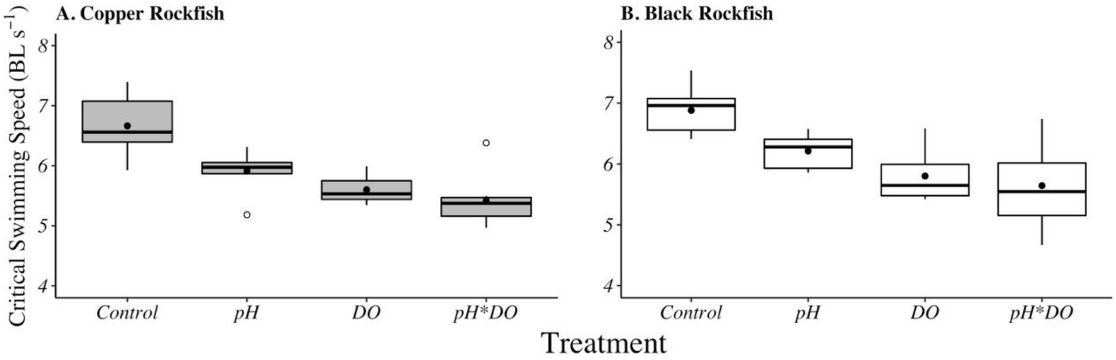

Figure 29. Left panel: Independent and combined effect of reduced pH and DO on critical swimming speed (U_{crit}) of juvenile copper rockfish (n=8 fish/treatment). A significant effect of reduced DO was detected (P = 0.018), while pH and simultaneous exposure to both stressors was not significant in the linear mixed effect model (P > 0.05). Right panel: Independent and combined effect of reduced pH and DO on U_{crit} of juvenile black rockfish (n=8 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on copper rockfish U_{crit} was detected in the linear mixed effect model (P > 0.05). U_{crit} for both species was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 30. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (length) on critical swimming speed (U_{crit}) of juvenile copper and black rockfish. U_{crit} for both species was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-values are marked in bold.

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	-0.798	0.301	4	-2.655	0.057
	DO 4.0	-1.093	0.294	4	-3.711	**0.021 ***
	pH 7.5 * DO 4.0	0.648	0.430	4	1.507	0.206
	Length	-0.028	0.042	23	-0.668	0.511
Black	pH 7.5	-0.821	0.470	4	-1.746	0.156
	DO 4.0	-1.248	0.469	4	-2.663	0.056
	pH 7.5 * DO 4.0	0.712	0.664	4	1.072	0.344
	Length	-0.020	0.0344	23	-0.591	0.560

<u>Ventilation rate.</u> There was no significant effect of pH, DO, or an interaction between the two stressors on copper rockfish ventilation rate (Figure 30, Table 31). For copper rockfish, mean ventilation rate increased in the reduced pH, reduced DO, and combined treatment by 11%, 16%, and 17%, respectively, above the ventilation rate of fish under control conditions. However, ventilation rate of black rockfish was significantly increased by the reduced DO treatment (Figure 30, Table 31). There was no significant effect of pH on black rockfish ventilation rate, and the interaction between DO and pH was also not significant. Mean ventilation rate increased in the reduced pH,

reduced DO, and combined treatment by 18%, 46%, and 44% respectively, above the ventilation rate of black rockfish under control conditions. Mean ventilation rates for both species across all treatments are listed in the Year 2: Comparisons Across Experimental Trials section.

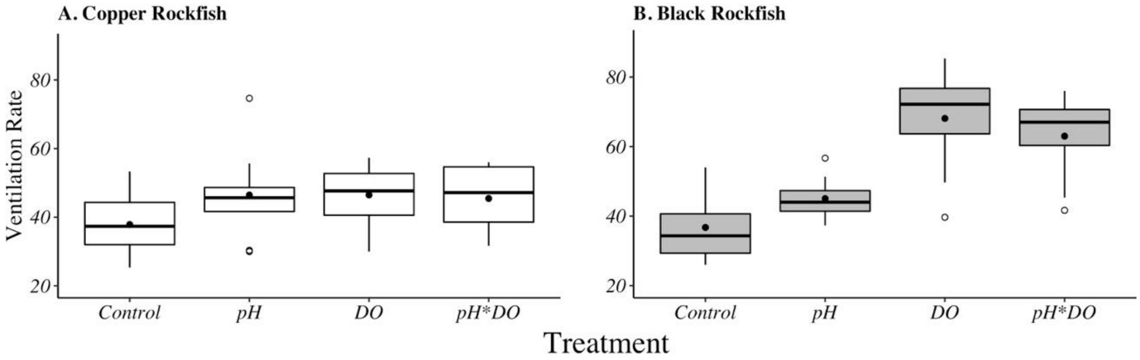

Figure 30. Left panel: Independent and combined effect of reduced pH and DO on ventilation rate (operculum openings per minute) of juvenile copper rockfish (n=9-10 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on copper rockfish ventilation rate was detected in the linear mixed effect model (P > 0.05). Right panel: Independent and combined effect of reduced pH and DO on ventilation rate (operculum openings per minute) of juvenile black rockfish (n=9-10 fish/treatment). A significant effect of reduced DO on black rockfish ventilation rate was detected (P = 0.004), while pH and simultaneous exposure to both stressors was not significant in the linear mixed effect model (P > 0.05). The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 31. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (length) on ventilation rate (operculum openings per minute) of juvenile copper and black rockfish. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-values are marked in bold.

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	8.492	4.629	4	1.835	0.141
	DO 4.0	6.761	4.652	4	1.453	0.220
	pH 7.5 * DO 4.0	-9.912	6.461	4	-1.534	0.200
	Length	0.646	0.400	30	1.612	0.117
Black	pH 7.5	8.584	5.228	4	1.642	0.176
	DO 4.0	31.908	5.236	4	6.094	**0.004 ***
	pH 7.5 * DO 4.0	-12.245	7.374	4	-1.660	0.172
	Length	-0.430	0.270	30	-1.590	0.122

Standard metabolic rate (SMR). Average DO concentrations measured throughout SMR trials for copper and black rockfish demonstrate that DO concentration remained stable and near the desired set point (Figures 32-33). However, temperature conditions differed substantially across trials, ranging between 10-14°C across SMR trials (Figures 34-35). pH conditions were spot checked prior and after SMR trials, but were not continuously monitored. There was no significant effect of pH, DO, or an interaction between the two stressors on standard metabolic rate (corrected for temperature) in copper rockfish (Figure 31, Table 32). While statistically insignificant, SMR in black rockfish demonstrated a 3% and 10% decline in the reduced pH and combined treatment (Figure 31, Table 32). Due to poor health and elevated mortality of black rockfish in the low DO treatment, results for SMR, MMR, or aerobic scope for black rockfish in response to the independent effect of reduced DO are not reported. Mean SMR for both species across all treatments are summarized in the *Year 2: Comparisons Across Experimental Trials* section.

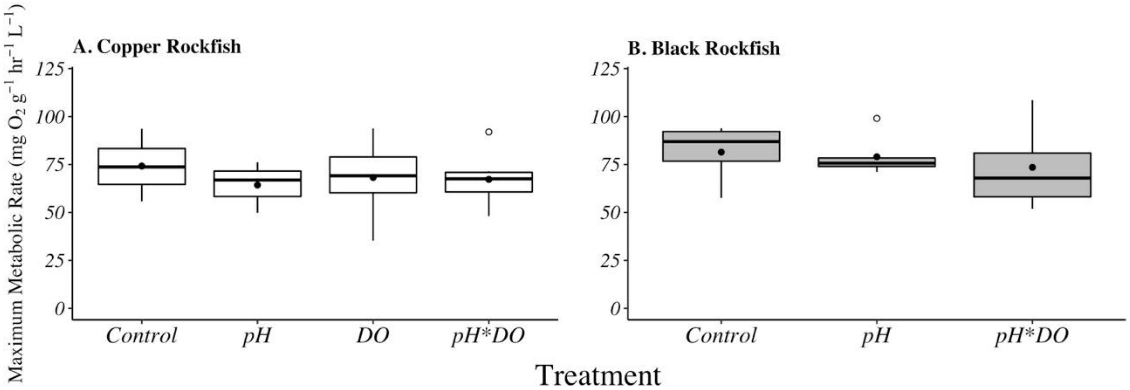

Figure 31. Left panel: Independent and combined effect of reduced pH and DO on standard metabolic rate (SMR) of juvenile copper rockfish (n=5-8 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on copper rockfish SMR was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent and combined effect of reduced pH and DO on SMR of juvenile black rockfish (n=2-5 fish/treatment). No significant effect of pH or simultaneous exposure to both stressors on black rockfish SMR was detected in the linear mixed effect model ($P > 0.05$). SMR for both species was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 32. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (weight) on standard metabolic rate (SMR) of juvenile copper and black rockfish. SMR for both species was scaled to a standard temperature (12 °C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	-7.386	12.372	3	-0.597	0.593
	DO 4.0	-7.782	9.897	3	-0.786	0.489
	pH 7.5 * DO 4.0	5.994	14.933	3	0.401	0.715
	Weight	-4.336	3.043		-1.425	0.176
Black	pH 7.5	-5.557	12.668	3	-0.439	0.691
	pH 7.5 * DO 4.0	-8.772	12.992	3	-0.675	0.548
	Weight	-2.723	2.422	9	-1.124	0.290

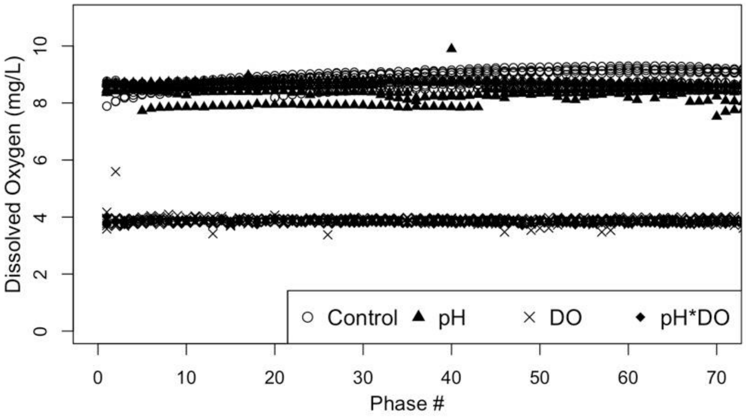

Figure 32. Average dissolved oxygen concentrations for each phase (measurement period) recorded during Year 2 SMR trials on copper rockfish. Each record applies to up to 4 fish being tested simultaneously.

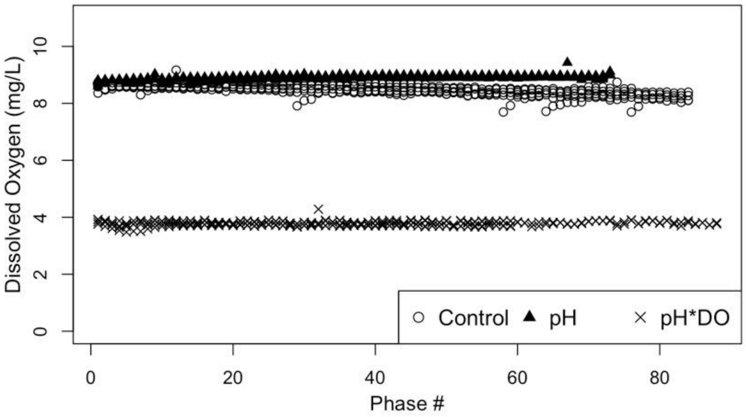

Figure 33. Average dissolved oxygen concentrations for each phase (measurement period) recorded during Year 2 SMR trials on black rockfish. Each record applies to up to 4 fish being tested simultaneously.

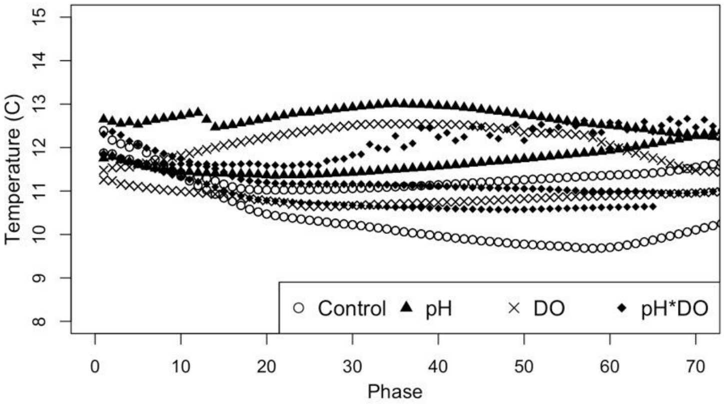

Figure 34. Average temperature concentrations for each phase (measurement period) recorded during Year 2 SMR trials on copper rockfish. Each record applies to up to 4 fish being tested simultaneously.

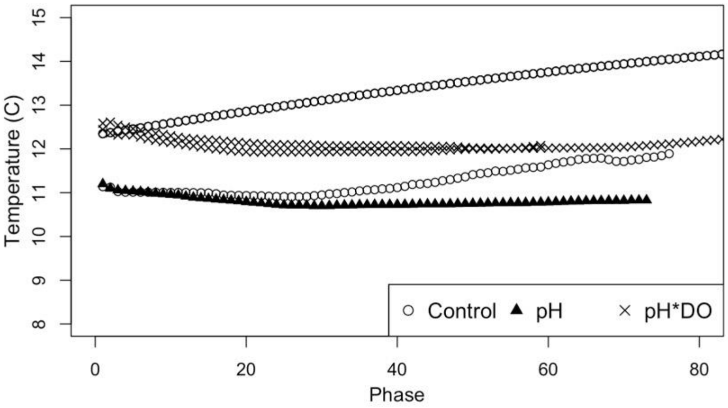

Figure 35. Average temperature concentrations for each phase (measurement period) recorded during Year 2 SMR trials on black rockfish. Each record applies to up to 4 fish being tested simultaneously.

Maximum metabolic rate (MMR). There was no significant effect of pH, DO, or an interaction between the two stressors on MMR in copper rockfish (Figure 36, Table 33). An effect of copper rockfish size (weight) on MMR was observed, reflecting a tendency for larger fish to exhibit increased MMR compared to smaller fish (P = 0.039, Figure 37). There was no significant effect of pH on black rockfish MMR and simultaneous exposure to reduced DO and pH was also not significant (Figure 36, Table 33). However, black rockfish did demonstrate a 37% decline in MMR in the combined treatment compared to control. Additionally, there was a significant effect of temperature on black rockfish MMR (P = 0.038). Mean MMR for both species across all treatments are summarized in the *Year 2: Comparisons Across Experimental Trials* section.

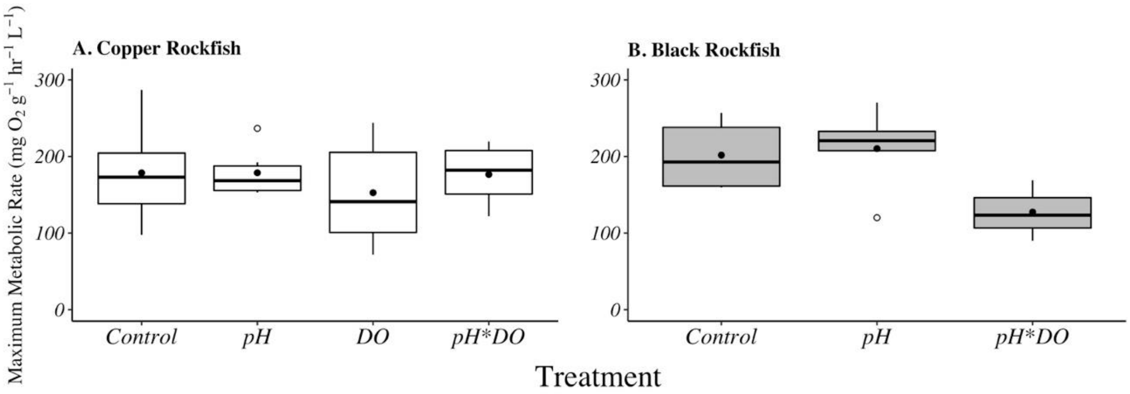

Figure 36. Left panel: Independent and combined effect of reduced pH and DO on maximum metabolic rate (MMR) of juvenile copper rockfish (n=4-6 fish/treatment). No significant effect of pH, DO, or simultaneous exposure to both stressors on copper rockfish MMR was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent and combined effect of reduced pH and DO on MMR of juvenile black rockfish (n=4-5 fish/treatment). No significant effect of pH or simultaneous exposure to both stressors on black rockfish MMR was detected in the linear mixed effect model ($P > 0.05$). MMR for both species was scaled to a standard temperature (12 °C) using a Q_{10} (=2) relationship. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 33. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO and fish size (weight) on maximum metabolic rate (MMR)) on juvenile copper and black rockfish. MMR for both species was scaled to a standard temperature (12°C) using a Q_{10} (=2) relationship. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML). Significant p-value are marked in bold.

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	20.031	30.710	4	0.652	0.550
	DO 4.0	-43.218	29.361	4	-1.472	0.215
	pH 7.5 * DO 4.0	16.150	43.563	4	0.371	0.730
	Weight	20.456	8.992	14	2.275	**0.039 ***
Black	pH 7.5	63.194	35.758	3	1.768	0.175
	pH 7.5 * DO 4.0	-55.054	39.652	3	-1.388	0.259
	Weight	1.495	4.737	5	0.316	0.765
	Temperature	-224.210	79.999	5	-2.803	**0.038 ***

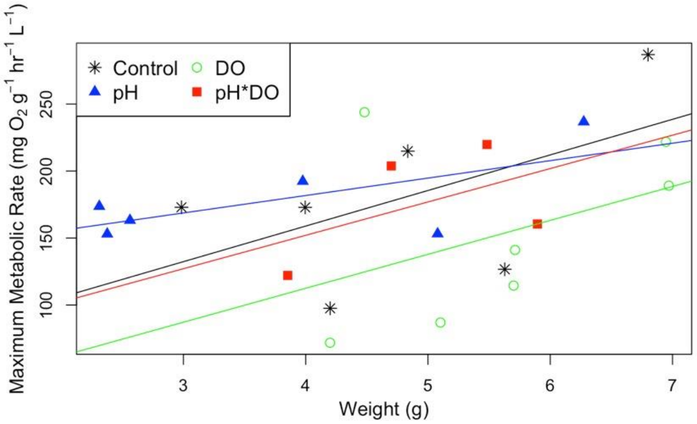

Figure 37. Effect of copper rockfish size (wet weight) on maximum metabolic rate (MMR) across the control, reduced DO, reduced pH, and combined (pH*DO) treatment.

Aerobic scope. Individual variability in aerobic scope was high and sample sizes were extremely limited. Since there are results from only one copper rockfish in the reduced pH treatment, the independent effect of pH was not included in the linear mixed effect model analysis. There was no significant effect of the reduced DO or combined treatment on copper rockfish aerobic scope (Figure 38, Table 34). There was no significant effect of pH or the combined treatment on black rockfish aerobic scope (Figure 38, Table 34). However, black rockfish demonstrated a 69% in aerobic scope in the combined treatment. Aerobic scope for both species across all treatments are summarized in the Year 2: Comparisons Across Experimental Trials section.

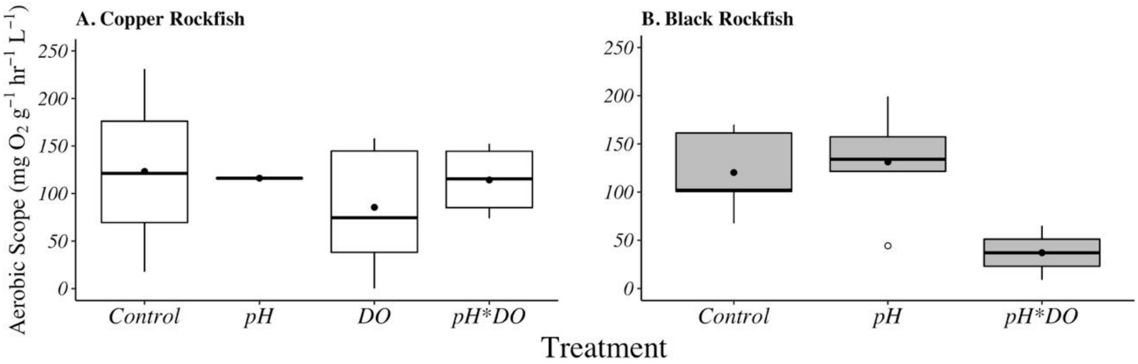

Figure 38. Left panel: Independent and combined effect of reduced pH and DO aerobic scope of juvenile copper rockfish (n=1-7 fish/treatment). No significant effect of DO or simultaneous exposure to both stressors on copper rockfish aerobic scope was detected in the linear mixed effect model ($P > 0.05$). Right panel: Independent and combined effect of reduced pH and DO on aerobic scope of juvenile black rockfish (n=4 fish/treatment). No significant effect of pH or simultaneous exposure to both stressors on black rockfish MMR was detected in the linear mixed effect model ($P > 0.05$). Aerobic scope for both species was scaled to a standard temperature (12°C) using a temperature coefficient (Q_{10}) = 2. The box spans the interquartile range, solid black segment shows the median value, black dot shows the mean value, and whiskers above and below the box show the lowest and highest datum still within 1.5 IQR of the lower and upper quartile. Outliers are indicated by open circles.

Table 34. Linear mixed effect model results for the fixed effects of reduced pH, simultaneous exposure to reduced pH and DO, and fish size (weight) on the aerobic scope of juvenile black rockfish. Aerobic scope for both species was scaled to a standard temperature (12°C) using a temperature coefficient (Q_{10}) = 2. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	DO 4.0	-50.386	37.976	2	-1.327	0.316
	pH 7.5 * DO 4.0	3.013	41.953	2	0.072	0.949
	Weight	40.712	14.985	8	2.717	**0.026 ***
Black	pH 7.5	9.189	52.334	2	0.176	0.877
	pH 7.5 * DO 4.0	-56.121	65.475	2	-0.857	0.482
	Weight	-7.802	8.269	6	-0.944	0.382

Critical oxygen tension (P_{crit}). There are no results to report for black and copper rockfish P_{crit} due to declining fish health over the course of experimental trials.

Somatic growth. Somatic growth rate was analyzed for a subset of individuals (n=8 fish/treatment) during the first 59-73 days in treatment. Somatic growth was not significantly affected by reduced pH, DO, or the interaction between the two stressors (Figure 39, Table 35). Growth rate was slower in black rockfish compared to copper rockfish. Growth rate was also slower in Year 2 copper rockfish compared to Year 1 copper rockfish.

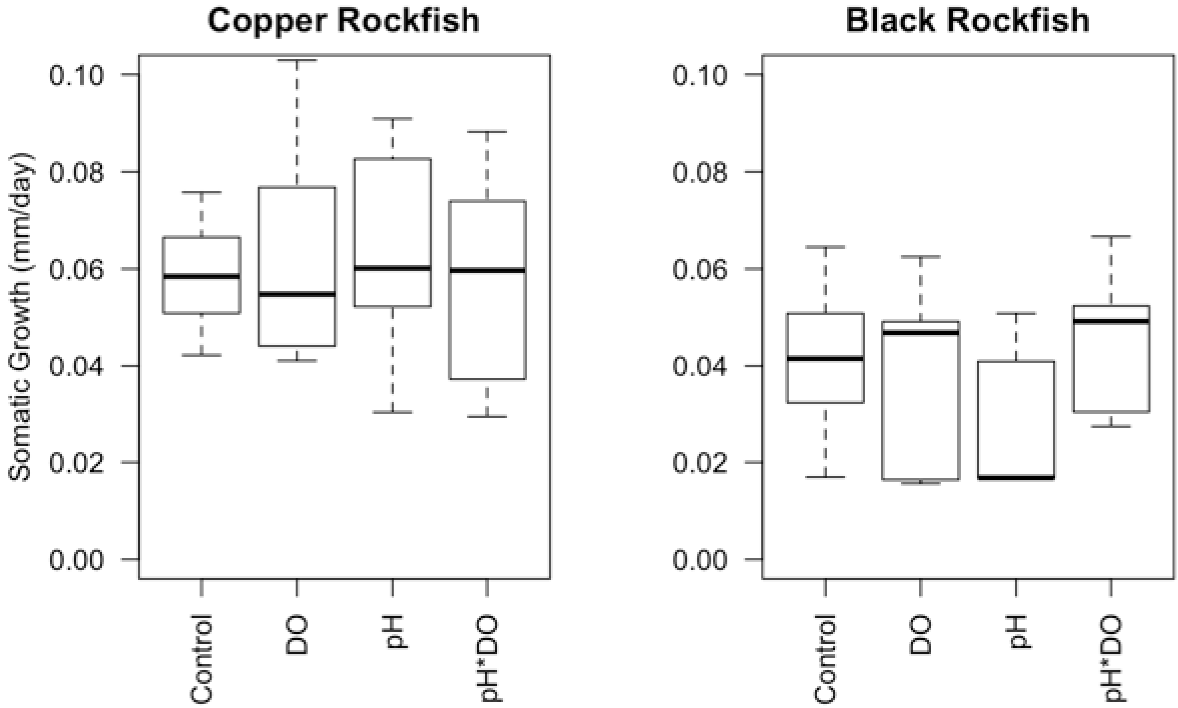

Figure 39. Left panel: Somatic growth rate (mm/day) for a subset of copper rockfish (n=8 fish/treatment), measured prior to treatment initiation and prior to U_{crit} trials (66-73 days in treatment). The box spans the interquartile range, solid black segment shows the median value, and whiskers above and below the box show the minimum and maximum values. Right panel: Somatic growth rate (mm/day) for a subset of black rockfish (n=8 fish/treatment), measured prior to treatment initiation and prior to U_{crit} trials (59-73 days in treatment). The box spans the interquartile range, solid black segment shows the median value, and whiskers above and below the box show the minimum and maximum values.

Table 35. Linear mixed effect model results for the independent and interactive effects of reduced pH and DO on somatic growth (mm/day) for juvenile copper and black rockfish for the first 59-73 days in treatments. The lme function automatically calculates t-tests and their associated p-values for fixed effects based on Restricted Maximum Likelihood (REML).

Species	*Fixed Effects*	*Estimate*	*Standard Error*	*DF*	*t-value*	*Pr (>|t|)*
Copper	pH 7.5	0.005	0.009	4	0.523	0.629
	DO 4.0	0.003	0.009	4	0.324	0.763
	pH 7.5 * DO 4.0	-0.001	0.009	4	-0.134	0.890
Black	pH 7.5	-0.014	0.009	4	-1.586	0.188
	DO 4.0	-0.003	0.009	4	-0.385	0.720
	pH 7.5 * DO 4.0	0.003	0.009	4	0.377	0.725

Year 2: Comparisons across experimental trials. Due to poor health and elevated mortality of rockfish as experiments progressed, the same subset of fish were not tested across all of the physiology trials. Thus, it was not possible to report individual progress across all trials. Overall, results from these experiments suggest that reduced DO had a stronger effect than pH on rockfish behavior and physiology, and dominated the effect of concurrent exposure to DO and pH stressors. Copper and black rockfish exposed to control conditions exhibited the greatest swimming capabilities and lowest ventilation rate. In addition, both species exposed to both reduced pH and DO concurrently demonstrated the worst swimming capabilities and lowest SMR. Ventilation rate of black rockfish significantly increased in response to reduced DO ventilation rate of copper rockfish remained unchanged. Copper and black rockfish behavioral responses (e.g.

change in escape time, relative lateralization, absolute lateralization) and physiological responses (e.g. swimming performance (U_{crit}), ventilation rate, standard metabolic rate (SMR), maximum, metabolic rate (MMR), aerobic scope, somatic growth (mm/day) during the first 59-73 days in treatment) across all treatments are listed in Tables 36-39.

Table 36. Mean escape time (seconds), relative brain lateralization (L_R), absolute brain lateralization (L_A), associated standard errors and sample size (N) for copper rockfish exposed to reduced pH, reduced DO, and simultaneous exposure to both stressors.

Treatment	*△ Escape Time (s)*	*Relative Lateralization* (L_R)	*Absolute Lateralization* (L_A)
Control	137.92 +/- 38.3 N = 13	-26 +/- 38.4 N = 20	36 +/- 8.0 N = 20
pH	140.55 +/- 42.4 N = 11	1 +/- 35.2 N = 20	27 +/- 6.0 N = 20
DO	-130.00 +/- 34.7 N = 14	14 +/- 30.5 N = 20	26 +/- 5.8 N = 20
*pH*DO*	-123.93 +/- 32.0 N = 15	-10 +/- 36.4 N = 20	30 +/- 6.7 N = 20

Table 37. Mean escape time (seconds), relative brain lateralization (L_R), absolute brain lateralization (L_A), associated standard errors and sample size (N) for black rockfish exposed to reduced pH, reduced DO, and simultaneous exposure to both stressors.

Treatment	*△ Escape Time (s)*	*Relative Lateralization* (L_R)	*Absolute Lateralization* (L_A)
Control	-296.06 +/- 69.8 N = 18	1 +/- 39.19 N = 20	31 +/- 6.9 N = 20
pH	63.74 +/- 14.6 N = 19	12 +/- 37.5 N = 20	32 +/- 7.2 N = 20
DO	-27.71 +/- 6.7 N = 17	15 +/- 31.0 N = 20	25 +/- 5.6 N = 20
*pH*DO*	-109.17 +/- 25.7 N = 18	-3 +/- 32.0 N = 20	23 +/- 5.1 N = 20

Table 38. Mean swimming performance (U_{crit}), ventilation rate (BPM), standard metabolic rate (SMR), maximum metabolic rate (MMR), aerobic scope, somatic growth rate (mm/day during the first 66-73 days in treatment), associated standard errors, and sample size (N) for copper rockfish exposed to reduced pH, reduced DO, and simultaneous exposure to both stressors.

Treatment	U_{crit}	*Ventilation rate*	*SMR*	*MMR*	*Aerobic scope*	*Somatic growth (mm/day)*
Control	6.66 +/- 0.17 N = 8	37.87 +/- 9.24 N = 10	74.23 +/- 8.14 N = 4	178.64 +/- 27.30 N = 6	123.31 +/- 61.62 N = 3	0.059 +/- 0.004 N = 8
pH	5.92 +/- 0.12 N = 8	46.44 +/- 13.49 N = 9	64.30 +/- 7.76 N = 3	178.68 +/- 13.07 N = 6	116.14 +/- NA N = 1	0.064 +/- 0.007 N = 8
DO	5.60 +/- 0.08 N = 8	46.50 +/- 8.44 N = 10	68.25 +/- 6.87 N = 8	152.74 +/- 25.30 N = 7	85.50 +/- 24.20 N = 7	0.062 +/- 0.008 N = 8
*pH*DO*	5.42 +/- 0.15 N = 8	45.47 +/- 9.71 N = 10	67.27 +/- 5.11 N = 7	176.55 +/- 22.05 N = 4	114.29 +/- 19.31 N = 4	0.057 +/- 0.008 N = 8

Table 39. Mean swimming performance (U_{crit}), ventilation rate (BPM), standard metabolic rate (SMR), maximum metabolic rate (MMR), aerobic scope, somatic growth rate (mm/day during the first 59-73 days in treatment), associated standard errors, and sample size (N) for black rockfish exposed to reduced pH, reduced DO, and simultaneous exposure to both stressors.

Treatment	U_{crit}	*Ventilation rate*	*SMR*	*MMR*	*Aerobic scope*	*Somatic growth (mm/day)*
Control	7.06 +/- 0.22 N = 8	36.74 +/- 9.00 N = 9	81.48 +/- 6.87 N = 5	201.76 +/- 19.84 N = 5	120.28 +/- 19.59 N = 5	0.041 +/- 0.005 N = 8
pH	6.21 +/- 0.10 N = 8	45.03 +/- 5.76 N = 10	79.04 +/- 4.15 N = 6	210.29 +/- 24.87 N = 5	131.29 +/- 25.47 N = 5	0.027 +/-0.007 N = 8
DO	5.80 +/- 0.15 N = 8	68.10 +/- 14.46 N = 10	NA	NA	NA	0.038 +/- 0.005 N = 8
*pH*DO*	5.64 +/- 0.20 N = 8	65.90 +/- 14.51 N = 10	73.52 +/- 10.05 N = 5	127.45 +/- 22.93 N = 3	37.06 +/- 28.11 N = 2	0.045 +/- 0.005 N = 8

DISCUSSION

This study is part of an investigation to better understand how long-term climate-driven changes in ocean pH and DO might impact the behavior and physiology of juvenile rockfish. The results of this study demonstrate that juvenile rockfish exhibit a variety of responses when exposed to reduced DO or pH, with behavioral trials generally providing little evidence of impact, in contrast to the more substantive responses resolved in several of the physiological measures of performance. Each of these sets of experiments are considered in greater detail below.

Behavioral Responses

Brain lateralization

Year 2 copper rockfish exhibited a significant population-level shift from right to left turning preference in response to reduced DO. The left-turn bias shown in the detour test suggests preferential right eye use (Bisazza et al. 1997). As hypothesized in Rogers (2010), exposure to DO stressor might cause a shift in rockfish information processing from the left to right brain hemisphere. Although studies on the effects of DO on brain lateralization are lacking, severe hypoxic conditions have been found to cause an increase in the frequency of staghorn sculpin individuals to a left-turning bias (Lucon-Xiccato et al. 2014), which matches my findings. Furthermore, there is evidence that exposure to hypoxic conditions can cause physiological disruptions in the teleost brain, such as upregulation of enzymes that can prevent protein synthesis in the brain and result in oxidative damage to brain cells (Rahman & Thomas 2015). Copper rockfish also exhibited a (non-significant) tendency towards left-turning in response to reduced pH, yet counterintuitively, copper rockfish demonstrated a tendency to turn towards the right, though with diminished consistency, when exposed to both stressors simultaneously. Interestingly, this pattern was also suggested (non-significantly) in results from lateralization trials for black rockfish. Recognizing the need for caution in any interpretation of non-significant results, these counterintuitive, yet repeated patterns raise interesting questions around the possibility that interaction between pH and DO somehow counters any independent effects of pH and DO on relative brain lateralization.

Both species displayed extremely high individual variability in absolute lateralization. While individual variation in the strength of brain lateralization is not surprising – it is a common observation across many species (Vallortigara & Rogers 2005) – it was expected that exposure to reduced pH or DO would weaken lateralization (reduce specialization) since several studies have demonstrated such an effect (Domenici et al. 2011, Jutfelt et al. 2013, Domenici et al. 2014, Lucon-Xiccato et al. 2014, Welch et al. 2014). Behavioral abnormalities in fish exposed to reduced pH, such as loss of behavioral lateralization and inappropriate olfactory preferences, have been effectively reversed by treatment with an antagonist of the $GABA_A$ receptor, strengthening the hypothesis that exposure to reduced pH may cause depolarization of the $GABA_A$ neurotransmitter receptor (Nilsson et al. 2012, Hamilton et al. 2014). While this study did not test for pH-induced changes in $GABA_A$ receptors, my results suggest that rockfish might effectively compensate for exposure to reduced pH through alternate mechanisms that minimize any effect on ion concentration gradients across neuronal membranes.

Escape response

During both years, rockfish exhibited high individual variability in escape response; some fish never escaped (spent >30 minutes in the chamber), while others escaped in a matter of seconds. Previous studies have demonstrated that exposure to reduced pH causes longer escapes times in stickleback (Jutfelt et al. 2011), as well as increased anxiety (preference for dark vs. light zones) in juvenile rockfish (Hamilton et al. 2014). Thus, longer escape times (reduced boldness) were expected in response to reduced pH and DO. However, rockfish in this study did not express significant changes in escape time (boldness) in response to either stressor. In fact, results demonstrate an unexpected trend of shorter escape times in Year 1 copper rockfish in response to reduced DO, where average escape time was 53% faster in 2.0 DO fish compared to control fish. This pattern is consistent with a behavioral response of fish seeking to escape hypoxic conditions. An initial increase in swimming activity in response to hypoxia has been observed in several species, presumably as an avoidance reaction to hypoxic waters (Domenici et al. 2013). Hypoxia tolerance in rockfish appears to vary across species, and is likely a function of the environment in which species have adapted to live in. Hypoxic conditions off the Oregon coast negatively affected copper rockfish movement, reducing their home ranges >30%, while quillback rockfish foray behavior remained unchanged (Rankin et al. 2013). Additionally, groundfish surveys within the CCS have demonstrated that spotted ratfish and petrale sole exhibited sensitivity to low oxygen availability, while greenstriped rockfish and Dover sole distributions were unaffected (Keller et al. 2015). Alternatively, rockfish in this study that did not display an escape behavior (remained in the chamber >30 minutes) may have been seeking shelter rather than seeking better available water conditions. Alternate escape response methods (e.g. escape response to predator stimulus) should be utilized to better understand the effect of DO and pH on juvenile rockfish escape behavior.

Physiological Responses

Critical swimming speed (U_{crit})

My results suggest that exposure to reduced DO has a more consistent, and possibly stronger influence on swimming performance in juvenile rockfishes than does exposure to reduced pH. These results, however, also suggest that the effect of exposure to reduced pH, while weaker, is fairly consistent, and that experiments capable of measuring responses for a larger sample size will have a strong likelihood of detecting and quantifying these effects. Furthermore, while the interaction terms were not significant, reduced swimming performance in the combined stressor treatments is consistent with an additive effect of pH and DO on swimming performance, and warrants further study.

There are no published studies to date that have investigated the interactive effect of pH and DO on U_{crit}, and studies on the independent effect of reduced pH on fish swimming physiology have shown inconsistent results. Studies focused on newly settled tropical clownfish (Munday et al. 2009), subtropical cobia larvae (Bignami et al. 2013), and juvenile Atlantic cod (Melzner et al. 2009) have all demonstrated insensitivity in U_{crit} to reduced pH. However, other research has demonstrated a negative effect of elevated reduced pH on swimming endurance in dolphinfish larvae (*Coryphaena hippurus*) (Pimentel et al. 2014), and on species-specific U_{crit} in juvenile rockfish (Hamilton et al. 2017). While copper and black rockfish in this study both showed reductions in swimming performance in response to reduced pH, the effect was not statistically significant at $\alpha = 0.05$. However, the observed reductions in swimming performance in response to pH demonstrate that rockfish physiology may be more vulnerable to OA than previously assumed. Acidification of the blood and respiratory tissue pigments can reduce oxygen uptake and delivery (known as "limiting stress"), which could have negative effects on swimming performance. Also, the cost of coping with ATP-hungry acid-base regulation can cause shifts in energy allocation (Heuer & Grosell 2014). Hypercapnia exposure has consistently been found to cause shifts in metabolic pathways in polar, temperate, and tropical fish species, with several reported changes in the metabolic profile of muscle tissue, which has been interpreted as a shift from aerobic to anaerobic metabolism in these tissues (Michaelidis et al. 2007, Strobel et al. 2012, Strobel et al. 2013a, Strobel et al. 2013b, Tseng et al. 2013). Changes in metabolic enzyme activity and regulatory genes in juvenile rockfish muscle tissue exposed to chronic pH 7.3 have also been demonstrated (Hamilton et al. 2017). Such changes in metabolic pathways can have profound effects on an individual's energy budget and allocation (Heuer & Grosell 2014). Muscle tissue samples were taken in this study for gene expression analysis, but the effect of hypercapnia on rockfish muscle tissue, from this study, remain unknown.

The insignificant pH and DO interaction suggests that reductions in rockfish swimming capability under combined exposure to low DO and low pH was dominated by the effects of low DO. The negative effects of oxygen depletion on swimming performance of freshwater and marine fish have been well documented using the U_{crit} protocol (Domenici et al. 2013, Dahlberg et al. 1968, Jones et al. 1971, Jourdan-Pineau et al. 2010, Fu et al. 2011, Herbert & Steffensen 2005, Brady et al. 2009, Zhang et al. 2010). However, acclimation period and treatment level vary greatly across studies, making it difficult to draw general conclusions about adaptation potential or physiological limits. The observed reductions in swimming capacity in response to reduced DO are assumed to be a consequence of limiting oxygen supply to the slow-twitch red muscles that enable prolonged swimming (Domenici et al. 2013). Results from this study demonstrate that long-term exposure to DO levels of 4.0 mg/L or less may be detrimental to rockfish swimming performance, suggesting that rockfish have limited ability to adapt to reduced DO environments. Preliminary results from a short-term exposure experiment (results not shown) suggest that these effects may manifest over relatively short time scales (1-4 hours) and increase over the course of long-term exposure. Any decline in juvenile rockfish swimming performance, if it manifests in natural systems as climate change progresses, has several obvious implications, including inability to escape predation, successfully forage, swim against currents, and settle to desirable habitats, which could have cascading effects on trophic interactions and population reproductive success.

Ventilation rate

Ventilation rate trials were only conducted in Year 2, and demonstrated species-specific ventilatory response to DO. Contrary to expectation, copper rockfish did not exhibit changes in ventilation rate in response to pH, DO, or the interaction between the two stressors. Black rockfish ventilation rate was also not effected by pH but was significantly higher in the reduced DO treatment (46% increase). Black rockfish ventilation rate was elevated in the combined treatment, but the interaction was insignificant, suggesting that the observed hyperventilation was largely (or entirely) in response to reduced DO. It is common for fish to adjust ventilation rates in response to reduced pO_2 levels (Gilmour 2001). Hyperventilation in response to reduced DO has been observed in the common sole (Cannas et al. 2012), dogfish (Metcalfe & Butler 1984, Perry & Gilmour 1996), starry flounder (Watters & Smith 1977), juvenile southern flounder (Taylor & Miller 2001), Atlantic cod (Kinkead et al. 1991), and numerous freshwater species (Gilmour 2001). However, a few species (red grouper and black sea bream) have demonstrated a reduction in operculum movement during hypoxia (Wu & Woo 1984), and others have demonstrated initial increase in ventilation rate followed by a decline in ventilation frequency as DO levels continue to decline (Tallqvist et al. 1999, McKenzie et al. 2002, Maxime et al. 1995).

The observed increase in black rockfish ventilation rate in response to low ? DO can have severe consequences for individual survival. First, an increased energy allocation to ventilation will likely cause a decline in other activities, such as searching for food or habitat, thus potentially affecting fish growth and survival. Hyperventilation can also increase vulnerability to predation due to increased visibility to predators and reduced energy allocation for escape responses, potentially forcing fish to hide rather than flee from predation (Kramer 1987, Domenici et al. 2013). The species-specific differences in ventilation rate suggest that black and copper rockfish may utilize different physiological mechanisms to cope with long-term reduced oxygen stress. Black rockfish are more active than copper rockfish; they are often found swimming in schools in the mid-water column while copper rockfish are generally found resting on the bottom (Love et al. 2012). Thus, black rockfish likely have higher metabolic demands and greater sensitivity to conditions that affect their oxygen uptake. Additionally, due to their more mobile behavior, black rockfish may be more likely to escape unfavorable water conditions than copper rockfish. Also, copper rockfish may utilize increased ventilation strategies that were not measured in this study, such as an increase in ventilation volume (expansion of oral-branchial cavities), which is considered more energy efficient than changes in ventilation rate (Perry et al. 2009). Additionally, some species can ameliorate the negative effects of oxygen depletion on performance through gill remodeling (Nilsson 2007, Wu & Woo 1985) or increasing blood hemoglobin concentration (Wood & Johansen 1972; Randall 1982; Val et al. 1995). To investigate whether either species copes with long-term oxygen depletion via these mechanisms, gill and hematocrit samples were taken from all individuals at the end of the experiment and sent to CSU Monterey Bay for analysis. Since these samples have not been analyzed, conclusions regarding gill remodeling or increased hematocrit concentration are to be determined.

Respiratory adjustments in response to reduced pH are sparse compared to the amount of research focused on the effects of reduced O_2 (Gilmour 2001). While many studies on marine and freshwater fish have found a similar hypervenilatory response to reduced pH as low O_2 (Perry & Gilmour 1996, McKendry 2001, Borch et al. 1993, Graham et al. 1990), many fish species have demonstrated no ventilation response to changes in seawater pH (McKendry 2001, Croecker & Cech 1998). It is presumed to be more cost effective for fish to utilize metabolic processes for acid-base regulation rather than respiratory adjustments. Thus, it appears juvenile rockfish may rely on the former to cope with reduced pH conditions.

Standard metabolic rate (SMR)

Interpretation of the respirometry trials from Year 2 requires special caution, due to challenges in condition maintenance and husbandry, also discussed in greater detail below and in the *Results: Fish Health* section. Standard metabolic rate (SMR) of copper rockfish in Year 1 was negatively correlated with DO, with ~20% reductions in the 6.0, 4.0 and 2.0 DO fish. However, SMR in Year 2 copper rockfish exposed to reduced DO was hardly reduced from control. Effects of pH on copper and black rockfish are not significant and unclear. Furthermore, it was expected that fish simultaneously exposed to reduced pH and DO would demonstrate the lowest SMR, but SMR in the combined treatment for both species was only slightly reduced from control.

A general assumption that the cost of coping with reduced pH (acid-base regulation, osmoregulatory, and cardiorespiratory adjustments) adds loading stress (contributes to the base cost of maintenance), thus increasing fish SMR (Heuer & Grosell 2014), has not been consistently borne out by research. Indeed, only a few studies have shown an increase in SMR in response to reduced pH (Enzor et al. 2013, Munday et al. 2009), and many others have shown no effect (Couturier et al. 2013, Munday et al. 2009, Strobel et al. 2012), or have reported a decrease in SMR (Rummer et al. 2013). If there is an effect of pH on rockfish SMR, it does not appear to be a strong effect and will require much larger sample sizes to detect.

Marine fish cope with reduced oxygen availability in several ways, such as by increasing respiration (ventilation) rate, the number of red blood cells, or the oxygen binding capacity of hemoglobin, or by decreasing metabolism, with the latter being considered the most energetically efficient method. Metabolic depression can prolong survival in hypoxic environments by reducing ATP use, and has been observed in a variety of species (Wu 2002). This study was unable to examine species-specific differences in SMR in response to reduced DO (due to high mortality of nutritionally stressed black rockfish prior to and during our respirometry trials, see below). However, since juvenile black rockfish are considered more active than copper rockfish, and more active species are expected to have higher metabolic rates, the hypothesis that black rockfish would exhibit higher SMR in response to reduced DO than copper rockfish warrants further study.

While only healthy individuals (i.e. those that did not display symptoms of vitamin C deficiency) were used in respirometry trials, it is possible that efforts to quantify pH and DO effects on fish respirometry were compromised by declining fish health. In addition, although estimates of oxygen consumption were corrected for the effects of temperature prior to estimating SMR, these calculations are based on the assumption that $Q_{10} = 2$, rather than a factor estimated specifically for oxygen consumption in juvenile rockfishes. Absent improved control of experimental temperature, an independent or *a priori* estimate of Q_{10} for juvenile rockfish should be developed to support future studies of metabolic response in these species. We did examine the analyses using a smaller Q_{10} factor (Q_{10} =1.6), and the results qualitatively were insensitive and conclusions remained unchanged. Moreover, the level of variability observed between individuals suggests that a greater number of replicate tanks and individual fish will be required to definitively resolve any metabolic responses to reduced pH and reduced DO in these species.

Maximum Metabolic Rate (MMR)

Acidification of the blood by hypercapnia exposure is thought to limit the ability of fish to uptake and deliver oxygen (known as “limiting stress”), thus reducing MMR (review: Heuer & Grosell 2014). However, results from this study suggest that rockfish may be resistant to changes in MMR, even after long-term exposure to greatly reduced pH. A survey of previous studies yields mixed reports on the effects of OA on MMR, ranging from reduced (Munday et al. 2009) to increased (Couturier et al. 2013, Rummer et al. 2002) MMR and includes cases where no change in MMR was detected (Peterson & Gamperl 2010). However, it is generally expected that MMR in fish will decline in response to reduced pO_2 (Fry 1971, Claireaux et al., 2000, McBryan et al. 2013, Norin &

Clark 2016). A decline in MMR was exhibited by copper rockfish in Year 1 in response to DO, but that decline was not significant at $\alpha = 0.05$ ($p = 0.06$), suggesting that this species might be able to maintain MMR over long-term exposures to pO_2 as low as 2.0 mg/L. Black rockfish should be tested over a broader range of (reduced) DO levels to determine whether MMR is further reduced at more extreme DO levels. Furthermore, interpreting estimates of MMR for black rockfish is confounded by changes in fish health during these trials; most individuals struggled to maintain swimming position during the 5-minute chase period, suggesting that black rockfish swimming capabilities had greatly declined since the U_{crit} trial.

There are several different methods for measuring MMR. While swim tunnel respirometry is the preferred method for species that readily maintain active swimming, the chase method is an optimal method for species that do not normally engage in prolonged, active swimming, and seemed appropriate for juvenile rockfish. Methods used in this study are consistent with recommendations for chase time of 3-5 minutes that leave fish nonresponsive and exhausted (Clark et al. 2013). Studies have shown that chase-method MMR can take several minutes or even hours to manifest (Clark et al. 2013), such as a reported delay of 6-8 hours in adult coho salmon (Clark et al. 2012). This does not appear to be the case in rockfish: repeated measurements of oxygen consumption made for a subset of individuals always indicated that oxygen consumption was greatest during the period immediately following the chase.

Aerobic scope

The hypothesis that limited oxygen supply would negatively impact fish aerobic scope has been supported by several studies (Farrell & Richards 2009), but estimates of aerobic scope from this study do not strongly corroborate this conclusion. While aerobic scope decreased by 28% in Year 1 copper rockfish exposed to 2.0 DO, it was not significantly at $\alpha = 0.05$ ($P = 0.07$). Due to the large discrepancies in the literature on the effect of pH on SMR and MMR, it is not surprising that there are conflicting reports on how reduced pH impacts fish aerobic scope. Evidence of an increase (Couturier et al. 2013, Rummer et al. 2013), decrease (Munday et al. 2009), and no apparent change in aerobic scope in response to reduced pH (Melzner et al. 2009) have all been reported. Since rockfish in this study exposed to reduced pH did not demonstrate changes in SMR or MMR, it is also not surprising that no response in aerobic scope was detected. These results are inconsistent with an earlier study that found exposure to reduced pH caused a decline in aerobic scope of juvenile copper rockfish (Hamilton et al. 2017). While my study used similar treatments and exposure durations as Hamilton et al. (2017), differences in MMR protocols might explain differences in reported aerobic scope. Hamilton et al. (2017) used oxygen consumption rates of fish swimming at its maximal rate (obtained from U_{crit} trials) in a swim tunnel respirometer over a 20-minute interval. In my study, fish were chased for 5-minues at a speed one body length below their maximal rate.

Aerobic scope is considered to be a measure of ‘whole-animal’ fitness and an important control or correlate on fish growth, reproduction, and swimming performance

(Fry 1971, Clark et al. 2013). Further work to estimate this parameter in juvenile rockfish and how it responds to reduced pH and DO is needed to verify how rockfish metabolic rate and energy allocation will be impacted by changes in ocean pH and DO, especially considering the contrast in results reported here and in Hamilton et al (2017).

Critical oxygen level (P_{crit})

The decline in P_{crit} of copper rockfish in response to reduced DO, although insignificant, suggests that long-term acclimation to reduced DO might enhance hypoxia tolerance in this species. These results are corroborated by reports in the literature that have also demonstrated decreased P_{crit} following acclimation under hypoxic stress (Fu et al. 2011, Dan et al. 2014, Routley et al. 2013, Rogers et al. 2016). However, there are also cases where no change in P_{crit} was observed after long-term exposure to reduced DO, such as in juvenile snapper (Cook et al. 2013) and post-smolt Atlantic salmon (Remen et al. 2013). There are conflicting reports on the effects of pH on P_{crit}; some studies have reported no effect (Cochran & Burnett 1996) while others have reported an increase in P_{crit} (Cruz-Neto & Steffenssen 1997, Rogers et al. 2015, Rogers et al. 2016). It is hypothesized that increased P_{crit} in fish exposed to reduced pH conditions is caused by reduced oxygen transport capacity due to respiratory acidosis (Heuer & Grosell 2014). In contrast to this expectation, no effect of pH on P_{crit} of juvenile copper rockfish was detected in this study. Results from this study suggest that copper rockfish might be able to regulate acid-base balances efficiently without compensatory reductions in oxygen transport capacity.

Most research on P_{crit} has focused on the effect of temperature, with a general finding that increased temperatures elevate P_{crit} (Rogers et al. 2016). We did not examine temperature sensitivity of P_{crit}, and instead corrected for assumed effects of temperature to reduce the chance that variability in temperature would confound our ability to resolve responses to our treatments. However, with expected increases in ocean surface temperature due to ongoing climate change, P_{crit} response in juvenile rockfish under future ocean temperature conditions should be investigated. It is also important to note that P_{crit} in this study was reported for SMR (since P_{crit} was conducted post-SMR trials), and is thus likely higher than would be P_{crit} reported for routine metabolic rate (RMR). Since P_{crit} reported for RMR is considered more ecologically relevant (Rogers et al. 2016), future studies should compare rockfish P_{crit} for SMR and RMR. In addition, a significant size effect on copper rockfish P_{crit} was detected, and is particularly evident in the control treatment fish, where larger fish demonstrated higher P_{crit}. This finding is contradictory to previous research that has reported a negative relationship between increasing body mass and P_{crit} (Rogers et al. 2016, Perna & Fernandes 1996, Sloman et al. 2006).

Somatic growth rate

Results from this study do not strongly corroborate previous findings of reduced growth in early life stages of juvenile fish in response to reduced pH and hypoxic conditions (Baumann et al. 2012, Chabot & Dutil 1999). While no significant effect of exposure to reduced pH or reduced DO on somatic growth rates of copper and black rockfish was detected, there is a suggestive trend of reduced somatic growth (both in length and mass) in copper rockfish over approximately two months of exposure to reduced DO treatment in the first experiment. However, somatic growth in response to either stressor is potentially compromised by malnutrition issues in the second experiment. The effect of pH and DO stressors on juvenile rockfish growth requires further investigation.

SUMMARY

As climate change progresses, the frequency and duration of upwelling events that bring low pH, low DO water to nearshore habitats are expected to increase (Bakun et al. 2015, Rykaczewski & Dunne 2010). In addition, long-term global changes in ocean pH and DO are expected to occur within the next couple hundred years (Caldeira & Wickett 2005, Kelling et al. 2009), with already documented reductions in near-shore pH and expansion of oxygen minimum zones within the CCS (Feely et al 2008, Chan et al. 2008). However, very few studies have investigated the potential interactive effect of these stressors on temperate fish. The results of this study provide evidence that future levels of pH and DO expected from climate change might have negative effects on juvenile rockfish physiology, including swimming performance and ventilation rate. In contrast, juveniles of both species appear to be behaviorally resilient to changes in pH and DO, as no response was detected in absolute brain lateralization or escape response. However, a significant shift in relative lateralization in response to DO suggests potential consequences of exposure to stressors for juvenile rockfish brain function. Responses to reduced pH and to concurrently reduced pH and DO suggested patterns that, although not statistically significant, were shared across species. Further study with larger samples sizes will be required to evaluate these apparent patterns, and if they are corroborated, this presents an interesting challenge to understand the mechanisms that underpin independent and concurrent effects of reduced pH and reduced DO on individual- and population-level lateralization. Furthermore, whether responses measured in this study to pH treatments are driven by elevation in $[H^+]$ or increase in pCO_2 is a concern and warrants further investigation.

It was expected that black rockfish would exhibit greater resilience to these oceanographic stressors than copper rockfish, due to potential differences in early life history exposure to pH and DO stressors. Juvenile black rockfish are one of the few rockfish species found to utilize tide pool habitats as nursery grounds, which indicates an increased resilience to low DO, low pH conditions since these habitats exhibit drastic fluctuations in both stressors. However, the most prominent difference between the species was in ventilation rate under stress, which provided evidence of greater tolerance in copper rockfish. However, differences in the effect of reduced DO on relative lateralization suggest that brain function in copper rockfish may be more susceptible than that of black rockfish to changes in water quality. Both species exhibited similar changes in swimming performance to both stressors and no significant changes in metabolic responses to pH. A lack of reliable measurements of metabolic responses in black rockfish to reduced DO due to declines in fish health prevents comparison between species. Hamilton et al. (2017) found significant changes in rockfish physiology and behavior in response to reductions in pH, with stark species-specific differences. Thus, the lack of species-specific behavioral and physiological responses to reduced pH detected in this study was surprising.

Discrepancies between this study and others might be explained by differences in study region. Northern California is characterized as having stronger upwelling events than central California, and has been described as an ocean acidification ‘hotspot’ (Feely et al. 2008). In fact, the extreme pH (pH 7.3) and DO (2.0 mg/L) conditions used in the first year of this study are already observed along the northern California coast during strong upwelling events. Since rockfish in this region evolved in an especially dynamic pH and DO environment, they might be better adapted to cope with reductions in pH than rockfish in regions further south or fishes in regions where upwelling is a much weaker factor (e.g. tropics, North Atlantic). However, evolutionary adaptations will likely have their limits, and due to the continued release of carbon dioxide into the atmosphere, there might be a tipping point at which rockfish can no longer adapt to changing ocean conditions. The role of transgenerational responses in rockfishes, in which maternal experience affects gene activity in offspring, to changes in DO and pH remains to be explored.

Some of the variability observed among individuals is no doubt due to genetic differences, as the fish collected from the field are almost certainly drawn from a mixed population of diverse parentage. Numerous studies have demonstrated that parental exposure to hypoxic or increased carbon dioxide conditions has epigenetic effects on fish morphology and physiology, and in several cases, mediates the impacts of these stressors on offspring (Ho & Burggren 2012, Miller et al. 2012, Murray et al. 2014). If this same effect is also true for rockfish, it would be expected that parental species that inhabit deeper water habitat characterized by more variable and reduced pH (e.g. black rockfish) will produce offspring better adapted for future changes in water conditions. Follow up studies might benefit from assessing responses within known sibling groups, although it is not clear that this is presently practical for juvenile stages of rockfishes. Alternatively, combining larger sample sizes with detailed genetic assays--perhaps in concert with sampling along latitudinal population gradients-- might provide deeper insight to sources of variability in individual responses and the scope for adaptation by rockfishes to ongoing changes in ocean DO and pH. Latitudinal comparisons across natural gradients of DO and pH might also provide some scope for evaluating transgenerational responses, that is, whether and how maternal exposure to low pH or DO affects the tolerance of offspring.

Over the range of conditions examined, DO proved to have a stronger effect on rockfish physiology than pH, suggesting that hypoxic events may be more detrimental to future populations than ocean acidification. Declines in rockfish home ranges and mass fish mortalities have already been reported off the coast of Oregon in response to anoxic conditions (Rankin et al. 2013, Chan et al. 2008). However, P_{crit} results from this study provide evidence that copper rockfish have the potential to increase their tolerance for hypoxic conditions after long-term exposure to reduced DO. Further research on the potential for alternative hypoxia-induced mechanisms, such as modifications to gill surface area and increased oxygen binding hemoglobin, will further elucidate the capacity for rockfish to adapt to sustained hypoxic conditions. It is also important to consider how other organisms within the nearshore environment will also be affected, such as predators and prey of rockfish, to better predict future species-interactions. Further knowledge is required to predict how physiological responses observed in individuals might affect growth and survival over time and subsequently scale up to population-level responses. Due to the ecological importance of juvenile rockfish within the California Current Large

Marine Ecosystem and economic value of rockfish in the U.S. Pacific Coast fishery, further research is critical to better understand species' sensitivities and potential resilience to changes in ocean pH and DO expected under ongoing global climate change.

Recommendations for Future Studies

The U_{crit} experiment proved to be the most successful trial for assessing physiological changes in response to pH and DO stressors. Both species demonstrated clear responses, trials were relatively easy to conduct, and results from these experiments are easily compared to other studies since the U_{crit} protocol is a standardized measure of maximal swimming performance. Larger sample sizes would improve emerging patterns of pH and DO effects on fish swimming capabilities. Respirometry trials, including SMR, MMR, and P_{crit}, were time consuming, inherently difficult to conduct, and proved that much larger sample sizes are necessary due to substantial individual variability. As mentioned earlier, while only healthy fish were selected for respirometry trials, there is potential that even those fish that passed muster for inclusion in the trial were not in ideal health due to vitamin C deficiency in Year 2. Thus, further research on the effects of OA and hypoxia on juvenile rockfish metabolic rate is necessary. It is also recommended that the effect of different fasting periods on SMR is tested prior to conducting SMR trials to ensure a fasting period of 48 hours is sufficient (Clark et al. 2013). Future studies should also consider investigating the interactive effect of temperature with pH and DO stressors on rockfish metabolic rate along with other physiological measures.

Alternate behavioral studies should be pursued to measure changes in rockfish boldness in response to pH or DO stressors, such as activity level in a novel environment and responsiveness to threatening and benign objects (White et al. 2013). Also, pre- versus post-treatment exposure brain lateralization trials should be considered. Since behavioral responses demonstrated high individual variability, larger samples are highly recommended.

As a final note on animal husbandry, this study showed that rockfish require a diverse, enriched diet to maintain long-term health. A diet consisting of either vitamin-enriched krill or nutritionally balanced pellets is recommended for future studies to avoid confounding fish health issues with effects from environmental stressors. Subsequent experiments on black rockfish have been conducted in which nutritional decencies appear to be resolved and provide opportunities for evaluating present results and establishing protocols for future studies.

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APPENDIX A

Appendix A: Distribution and changes in fish size across treatments and trials (mean total length, total length standard deviation (sd), min total length, max total length, mean weight, mean weight standard deviation (sd) and sample size (N) for Year 1 copper rockfish.

Trial	*Treatment*	*TL*	*TL sd*	*min TL*	*max TL*	*Weight*	*Weight sd*	*N*
	CONTROL	50.8	6.6	39.00	59	1.83	0.750	20
INITAL	DO2.0	50.5	5.1	40.00	60	1.80	0.598	20
	DO4.0	52.4	5.2	43.00	59	1.91	0.603	20
	DO6.0	51.8	5.5	37.00	60	1.93	0.558	20
	PH7.2	51.2	6.6	40.00	60	1.85	0.721	20
	PH7.5	52.5	6.0	42.00	62	1.91	0.726	20
	PH7.8	52.0	5.0	42.00	61	1.95	0.623	20
ESCAPE	CONTROL	54.2	5.8	2.76	42	65.00	1.172	20
	DO2.0	54.9	5.7	2.52	43	63.00	0.847	19
	DO4.0	56.3	4.7	2.80	46	62.00	0.713	19
	DO6.0	57.6	5.8	3.01	42	65.00	0.897	20
	PH7.2	58.5	6.4	3.18	48	68.00	0.992	20
	PH7.5	58.9	6.2	3.14	49	68.00	1.050	20
	PH7.8	60.0	5.3	3.26	50	70.00	0.904	20
BL	CONTROL	59.3	6.6	47.00	70	3.28	1.136	20
	DO2.0	56.6	7.5	44.00	67	2.97	1.146	10
	DO4.0	59.7	5.0	50.00	67	3.29	0.863	20
	DO6.0	61.3	5.7	47.00	69	3.45	0.960	20
	PH7.2	60.5	6.8	49.00	70	3.56	1.145	20
	PH7.5	61.0	5.9	51.00	70	3.66	1.126	20

Trial	*Treatment*	*TL*	*TL sd*	*min TL*	*max TL*	*Weight*	*Weight sd*	*N*
	PH7.8	60.5	5.7	50.00	74	3.63	1.06	20
UCRIT	CONTROL	63.0	3.6	59.00	69	3.87	0.60	7
	DO2.0	61.8	4.2	57.00	68	3.81	1.01	6
	DO4.0	64.9	1.2	63.00	66	4.51	0.30	7
	DO6.0	65.1	3.0	59.00	68	4.41	0.78	8
	PH7.2	64.9	3.4	58.00	69	4.57	0.94	8
	PH7.5	64.8	2.4	60.00	67	4.46	0.67	8
	PH7.8	62.9	3.3	59.00	69	4.22	0.59	10
Final	CONTROL	75.7	6.3	62.00	85	12.02	3.39	16
	DO2.0	67.8	12.4	50.00	85	9.23	5.09	8
	DO4.0	75.5	6.8	60.00	84	12.14	3.10	19
	DO6.0	75.3	9.6	50.00	97	12.05	4.28	20
	PH7.2	72.3	6.4	64.00	87	10.54	2.74	18
	PH7.5	73.9	5.4	67.00	85	11.30	2.60	19
	PH7.8	72.2	8.4	50.00	88	10.72	3.97	19

APPENDIX B

Appendix B: Distribution and changes in fish size across treatments and trials (mean total length (TL), total length standard deviation (sd), min total length, max total length, sample size (N) for Year 2 copper rockfish.

Trial	*Treatment*	*TL*	*TL SD*	*Min TL*	*Max TL*	*N*
ESCAPE	CONTROL	58.2	3.9	52	64	13
	PH	60.4	3.8	55	69	14
	DO	58.9	3.7	55	68	14
	CROSS	57.8	3.0	53	64	11
BRAIN LAT	CONTROL	60.5	4.6	55	75	20
	PH	59.1	3.9	51	70	20
	DO	57.9	3.4	50	64	20
	CROSS	61.0	3.9	56	69	20
UCRIT	CONTROL	60.6	2.5	57	64	8
	PH	60.8	1.2	59	62	8
	DO	59.6	1.2	58	62	8
	CROSS	58.9	1.5	57	61	8
VENTILATION	CONTROL	62.2	3.3	58	68	10
	PH	65.7	3.8	63	74	10
	DO	65.1	5.7	61	80	10
	CROSS	62.3	3.8	58	70	9

APPENDIX C

Appendix C: Distribution and changes in fish size across treatments and trials (mean total length (TL), total length standard deviation (sd), min total length, max total length, sample size (N) for Year 2 black rockfish.

Length	*Treatment*	*TL*	*TL sd*	*Min TL*	*Max TL*	*N*
ESCAPE	CONTROL	76.9	7.2	68	91	19
	PH	75.1	7.9	61	90	19
	DO	77.0	6.4	69	90	19
	CROSS	75.7	4.7	66	83	20
BRAIN LAT	CONTROL	77.5	7.0	69	92	20
	PH	78.2	6.3	70	92	20
	DO	77.7	5.3	68	88	20
	CROSS	75.2	7.8	62	90	20
UCRIT	CONTROL	75.6	3.2	71	80	8
	PH	78.4	2.7	73	82	8
	DO	76.0	3.0	72	80	8
	CROSS	76.8	1.8	74	80	8
VENTILATION	CONTROL	82.2	6.4	74	94	9
	PH	80.1	8.4	69	96	10
	DO	83.5	7.1	74	96	10
	CROSS	82.9	6.3	73	98	10