AN ABSTRACT OF THE DISSERTATION OF

Alena L. Pribyl for the degree of Doctor of Philosophy in Fisheries Science presented on February 9, 2010.

Title: A Macroscopic to Microscopic Study of the Effects of Barotrauma and the Potential for Long-term Survival in Pacific Rockfish

Abstract approved:

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Carl. B. Schreck Steven J. Parker

Depleted species of rockfish (*Sebastes* spp.) from the Northeast Pacific experience high discard mortality due to "barotrauma," induced from the rapid change in pressure during capture. Research suggests rockfish have the potential to survive barotrauma if immediately recompressed, but the potential for long-term recovery is unknown. In this project, we studied the injuries that occur in rockfish during barotrauma and the potential for rockfish to recover from these injuries using a macroscopic to microscopic to molecular approach. We first assessed multiple species of rockfish for macroscopic and tissue-level injuries as a result of barotrauma; these injuries tended to be species-specific and included ruptured swimbladders, emphysema in the heart ventricle, emboli in the rete mirabile, and emboli in the head kidney. Next we investigated the potential for longer-term recovery in black rockfish (*S. melanops*) that underwent simulated decompression from 4.5 ATA (35 m depth) and subsequent recompression using hyperbaric pressure chambers. We assessed recovery over a 31 day period at three different time points at the macroscopic level, tissue level, blood level, and molecular level. Macroscopic and tissue

level injuries included ruptured swimbladders, which were slow to heal in some fish, and injury to the rete mirabile. At the blood level, we found no differences between treatment and control fish. At the molecular level, we created a rockfish-specific cDNA microarray to search for genes that might be differentially regulated as a result of barotrauma. We identified six genes from the innate immune system that were up-regulated at day 3 in treatment fish but were no longer up-regulated at day 31. In conclusion, recompressed rockfish will not be as competent as uncaptured rockfish because of unhealed swimbladders, injury to the rete mirabile, and up-regulation of the innate immune system. However, even rockfish that are injured will have a chance at survival that they would not have if they were not recompressed. The resumption of feeding, the ability of most swimbladders to hold gas again, the lack of a difference in blood chemistry measures between treatment and control fish, and the return of immune genes to neutral regulation are all good indicators that many black rockfish do have the potential for recovery.

A Macroscopic to Microscopic Study of the Effects of Barotrauma and the Potential for Long-term Survival in Pacific Rockfish

by
Alena L. Pribyl

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented February 9, 2010
Commencement June 2010

Doctor of Philosophy dissertation of Alena L. Pribyl presented on February 9, 2010

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Alena L. Pribyl, Author

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to my two major professors, Carl Schreck and Steve Parker, for providing me with the opportunity to return to school in marine fisheries and for providing unwavering support and encouragement while I explored the fields of histology, physiology, statistics and molecular biology. Their insightful suggestions and instructive comments contributed greatly to the evolution of my research and development as a scientist. A graduate student could not have asked for two better mentors.

I would like to thank my graduate committee members Virginia Weis, Michael Kent, and Cliff Pereira for their support and encouragement and their invaluable help and advice as I worked through the molecular biology, histology, and statistics portions of my research. Virginia mentored me in my first research project as an undergraduate and as a PhD student she invited me into her lab again for two-plus years as I worked through the molecular biology portions of my project; I am very appreciative of all the time and assistance she has invested in me. Mike opened up his lab to me for analysis of histology slides and provided invaluable assistance in identifying and explaining tissue abnormalities. Cliff took the time to really understand my data and provided excellent statistical advice. I was incredibly fortunate to have such a wonderful committee to assist me throughout my project.

I would like to thank my Graduate Council Representative, Dennis Hruby, for helpful comments and suggestions throughout the years. I would also like to thank Dennis's last-minute replacement for my defense, Lori Cramer, for being willing to act as a substitute on such short notice. I would also like to thank John Vansickle, for also being willing to act as a last-minute substitute to replace Cliff Pereira during my defense and for his statistical advice.

I would like to thank all the personnel at The Oregon Department of Fish and Wildlife (ODFW) in Newport, OR who spent three years assisting me in fish collections, fish husbandry, and sampling. I would especially like to thank Polly Rankin for fish collections, teaching me fish husbandry, and teaching me how to operate the hyperbaric pressure chambers. I would also like to thank Bob Hannah for his support and advice throughout this project. This project would not have been possible without the tremendous logistical and financial support of ODFW.

I would like to thank all of the charter boats who assisted me in fish collections, including *Endeavor*, *Misty*, *Miss Raven*, *Sampson*, *Tacklebuster*, and *Ilwaco Indian*.

I would like to thank Mark Camara and his lab for providing me with lab space for RNA extractions. I would also like to thank the Hatfield community for incredible support, encouragement and much needed distraction during my stay there. It was wonderful to be a part of the Hatfield community for the first three years of this project.

I would like to thank Wendy Phillips, Christy Schnitzler, Paul Lang, Tracey Momoda, and Caprice Rosato for helping me learn the molecular biology techniques I needed for this project. I would like to thank Grant Feist and Carisska Anthony for teaching me cortisol assays, and histology, respectively. I would also like to thank Jen Ramsay, Tracey Momoda, and Ben Clemens for lab assistance, support, and encouragement. I could not have asked for a better lab family! I would also like to thank my friends Judith Jobse, Betsy Glenn, Kate Boersma, Kerry Grimm, Suzanne Moellendorf, Allison Evans, and Sarah Ghasedi for helping keep me sane throughout the years.

I would like to thank the Department of Fisheries and Wildlife for providing a supportive and encouraging atmosphere in which to learn and work. I would also like to thank the office staff for their patience and assistance in helping me figure out forms and grant budgets.

I would like to thank all the organizations who financially supported this project including the Coastside Fishing Club (San Francisco, CA), ODFW (Newport, OR), the Hatfield Marine Science Center (Newport, OR), the Oregon State University (OSU) Foundation, the OSU Department of Fisheries and Wildlife, the Oregon Chapter American Fisheries Society, the Oregon Cooperative Fisheries Research Unit, and the National Oceanic and Atmospheric Administration's Saltonstall-Kennedy Grant.

I would like to thank my Foul Weather Friends, Paul Henderson, Mandy Matsuda, and Ngan Vo for introducing me to mountaineering and providing a wonderful balance of work and play. I would like to thank my college girlfriends Kristi Rietz, Erin Simonsen, and Melissa Yamamoto for sticking with me for so long and for some fabulous wine dinners over the years. I would also like to thank Christian Rinke for his incredible support and encouragement and for bringing humor and new adventure to my life.

Finally, I would like to thank my parents, Karel and Althea Pribyl, for providing unwavering support in all of my life decisions, and especially when they learned I was returning to school for yet another five years (and not even in a high-paying field)! I would also like to thank my brother, Karel Pribyl, and his family Jenny, Kailey, Maddie, and Josie for their support and encouragement.

CONTRIBUTION OF AUTHORS

Chapter 1: Steven Parker and Carl Schreck assisted with the project design and data interpretation. Steven Parker also obtained the necessary permits for fish collections, helped coordinate charter boats, assisted with fish collections and with sampling. Michael Kent assisted with the identification of tissue injury due to barotrauma from histology slides.

Chapter 2: Steven Parker and Carl Schreck assisted with the project design. Steven Parker also obtained the necessary permits for fish collections, and helped coordinate charter boats. Michael Kent assisted with the interpretation of tissue injury due to barotrauma, and the identification of parasites from histology slides.

Chapter 3: Steven Parker and Carl Schreck assisted with the project design, sampling, and data interpretation. Steven Parker also obtained the necessary permits for fish collections, helped coordinate charter boats, and assisted with fish collections. Michael Kent assisted with the identification of tissue injury due to barotrauma from histology slides. Kevin Kelley's lab assisted by running assays for insulin-like growth factor-1 on blood plasma samples.

Chapter 4: Virginia Weis, Carl Schreck, and Steven Parker assisted with the project design. Virginia Weis also assisted by donating lab space and expertise for the duration of the project. Carl Schreck also assisted with data interpretation.

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General Introduction

Rockfish background

The rockfish genus (*Sebastes*) is an ecologically diverse group of temperate marine fish that occur worldwide, with the majority of species (over 65 species) occurring in the North Pacific and Gulf of California (Love et al. 2002). As a result of this diversity, rockfish species have specialized to inhabit areas from nearshore to off the continental shelf, with both benthic and semi-pelagic life histories. Adult rockfish range in size from less than 20 cm in length in the pygmy rockfish *Sebastes wilsoni*, to 120 cm in length in the shortraker rockfish *S. borealis* (Love et al. 2002).

Rockfish are also long-lived with most species maximum longevity between 20 and 100 years. Some of the oldest known vertebrates are rockfish; for example the rougheye rockfish *S. aleutianus* and shortraker rockfish, captured in Alaskan waters, have been aged up to 205 and 157 years, respectively (Love et al. 2002). Species that live in colder temperatures (northerly species and bottom-dwelling species) tend to live longer than species that live in warmer temperatures (southerly species and mid-water species) (Love et al. 2002). In addition, the more long-lived a rockfish is, the longer it takes to mature. Shorter-lived semi-pelagic rockfish species will generally reach maturity between 4-5

years of age, while longer-lived demersal species will generally reach maturity between 10-15 years of age (Love et al. 2002). Research has also shown that in some species older female rockfish will produce greater numbers of high quality eggs compared to younger females, and thus have greater numbers of larvae surviving (Berkeley et al. 2004, Bobko and Berkeley 2004, Sogard et al. 2008). Because reproduction may be dependent on older females and not newly mature females, generation times for rockfish may even be longer than 5 to 15 years.

Barotrauma in rockfish

Many rockfish species are captured in commercial and recreational fisheries. Since the 1940s, when the balloon trawl was developed, many rockfish fisheries have experienced huge booms in fishing and subsequent collapses. In the 1960's, catch of Pacific ocean perch *Sebastes alutus* in the Gulf of Alaska reached over 462,664,217 kg. After the collapse of the Pacific ocean perch fishery, an expansion to other rockfish fisheries and further technological advancements in fishing led to a dramatic increase in rockfish landings along the entire West Coast from the 1970s to 1980s. Total catch along the west coast of North America peaked in 1989 at 99,790,321 kg (Love et al. 2002). Catches have been in decline ever since as allowable catch was reduced by federal and state managers. Currently seven species of rockfish off the West Coast are classified as "depleted" by the Pacific Fishery Management Council (PFMC 2008). As a result of

long generation times and sporadic recruitment, recovery of rockfish populations is expected to be slow.

Rockfish often occur in mixed-species assemblages, making it difficult for fishermen to target only a single species. As a result, bycatch of non-target rockfish species is a common occurrence. In the commercial fishery, most rockfish are captured using trawls; longlines and other fixed gears are also used, but to a lesser extent. Rockfish captured as bycatch using these methods are often severely injured by the time they reach the surface due to abrasions and crushing from other fish and the long period in the net or on a hook. In the recreational fishery, rockfish are captured using hook and line. Management effort controls in the recreational fishery currently include bag limits, size limits, gear restrictions, time/area closures and discard of species of concern (2009). Discard is necessary so depleted species will not be targeted, but often discard mortality of rockfish is high because as rockfish are captured and undergo a forced ascent, the pressure change can cause barotrauma and complications due to barotrauma (excessive buoyancy and or shock) which prevent successful release.

Barotrauma is a condition that results from swimbladder gas expanding as fish are brought up from depth (forced decompression). According to Boyle's Law, as pressure decreases, gas expands exponentially. Rockfish experience barotrauma because they are physoclists, which means they have a closed swimbladder (unlike physostomes such as

salmonids, whose swimbladder is connected to their esophagus via a duct, allowing uptake/release of gas through the mouth). As a rockfish undergoes forced decompression, the expanding gas inside the swimbladder often leaks into the visceral and cranial cavities; this is illustrated in Hannah et al. (2008b) by the development of bulges and air bubbles that were externally visible in rockfish during inflation of an intact swimbladder. The excess swimbladder gas can result in bloating, a ruptured swimbladder, crushed organs, eversion of the esophagus, exopthalmia, and excessive buoyancy (Gotshall 1964, Hannah and Matteson 2007, Hannah et al. 2008a, Jarvis and Lowe 2008, Rummer and Bennett 2005).

Barotrauma differs from other gas-related problems such as gas bubble disease in fish or decompression sickness in humans because the source of the gas is internal instead of external. Gas bubble disease is a condition that occurs in fish exposed to water supersaturated with dissolved gases, thus the source of gas is external. Gas bubble disease can result in a variety of injuries ranging from gas bubbles or blisters on external surfaces of fish, to exopthalmia, to emboli in the blood and tissues (Weitkamp and Katz 1980). Decompression sickness in humans results from divers breathing compressed air at depth and saturating their blood and tissues with gas. Although a diver will breathe the same volume of air at 30 m depth as at the surface, the number of air molecules taken in at 30 m of depth is four times the amount taken in at the surface because as pressure increases, the volume of air becomes compressed and more concentrated (PADI 2008).

As a diver ascends to the surface, if they do not allow enough time for excess gas to dissipate from their blood and tissues, the gas will come out of solution and form emboli. In barotrauma, the source of gas is the swimbladder. It is excess swimbladder gas escaping into the body cavity that causes barotrauma injuries. The blood and tissues of fish are not usually saturated with gas because the concentration of gas dissolved in seawater at depth is not usually at saturation (unless there is a phytoplankton bloom). The primary mechanism for most gases to dissolve in seawater is through atmospheric exchange (Emerson and Hedges 2008), thus the concentration of dissolved gas at depth rarely exceeds the concentration of dissolved gas at the surface.

Excessive buoyancy resulting from expanded swimbladder gas makes it difficult for many rockfish species to submerge on their own. Discarded rockfish are often left floating on the surface where they succumb to predation by birds, and/or thermal shock (Jarvis and Lowe 2008).

Recompression is a technique that has been used by some recreational fishermen to return depleted species of rockfish back to depth. Recompression involves any method that will help a rockfish overcome its buoyancy to submerge below the surface of the water. If a rockfish can submerge close to its original depth of neutral buoyancy, the expanded gases will compress again, relieving the fish of its excessive buoyancy. Devices that are often used to recompress rockfish include barbless, weighted hooks and weighted cages

(Theberge and Parker 2005). Venting is another method used to relieve rockfish of excessive buoyancy and involves puncturing the swimbladder with a syringe. However, for people unschooled in proper technique, this procedure can lead to puncturing of internal organs, and can introduce bacterial infections if the syringe is not sterile (Kent et al. 2005). We feel using a recompression device is a better option because it is less likely to cause further injury to the fish.

Recent studies have shown recompression devices are effective at increasing the short-term survival of rockfish with barotrauma (Hannah and Matteson 2007, Jarvis and Lowe 2008), however little is known about the long-term survival of recompressed rockfish. Thus far, only one study has investigated long-term survival in black rockfish, however the focus was on delayed mortality and the healing rate of swimbladders (Parker et al. 2006). No study has undertaken a complete macroscopic to microscopic evaluation of recovery in rockfish after recompression.

Rockfish swimbladder

The swimbladder in fish originated as a dorsal outgrowth of the gut used as a lung in primitive Osteichthyes (Alexander 1966). The original function was eventually lost, and the outgrowth became a buoyancy organ. In physostome fish, the swimbladder still retains a connection to the esophagus via a duct. In physoclist fish, which include rockfish, the connection to the esophagus is lost during embryonic development.

The rockfish swimbladder consists of two chambers; a secretory chamber and a resorption chamber, separated by an oval sphincter, that can be opened or closed as needed by vasoconstriction (Alexander 1966). For a physoclist fish to increase its buoyancy, it must increase the volume of gas in the swimbladder. The secretory chamber is where gas is deposited into the swimbladder via diffusion from blood capillaries of the rete mirabile and gas gland. The rete mirabile is a gas concentrating organ that consists of a large network of venous and arterial capillaries. Through countercurrent diffusion, gas from the venous capillaries diffuses into the arterial capillaries. Blood then flows from the rete into the gas gland where secretions of lactic acid and carbonic anhydrase from the gas gland, along with the Root and Bohr effect, help increase the dissolved gas partial pressures even more (Evans 1998). The high partial pressure of dissolved gas in the gas gland then diffuses into the secretory portion of the swimbladder and inflates it. When a rockfish wants to decrease its buoyancy, it must remove some of the gas from its swimbladder. This is done by opening the resorption part of the swimbladder via the oval sphincter. When the oval sphincter is opened, gas from the secretory section of the swimbladder can enter the resorption section of the bladder. The resorption chamber is highly vascularized and when gas enters the chamber, it is resorbed by blood vessels through diffusion (Evans 1998).

Research on rockfish reveals it takes longer to secrete gas into their swimbladder than it takes to resorb it from their swimbladder (McElderry 1979). This study also found that different species of rockfish have different rates of gas secretion and resorption. Starting at surface pressure (1.0 ATA), black rockfish *S. melanops*, which are semi-pelagic, were able to acclimate to a pressure of 4.0 ATA (approximately 30 m depth) in approximately 100 hours, whereas China rockfish *S. nebulosus*, a primarily benthic species, took about 250 hours to acclimate to the same increase in pressure. Resorption rates were also different; black rockfish took less than 30 min to make a 10% reduction in buoyancy whereas China rockfish took 2 hours to make a 10% reduction in buoyancy. Although resorption of gases is faster than secretion of gases in rockfish, according to this rate of resorption, it would still take approximately 13 hours to bring a black rockfish from a depth of 30 m to the surface. The rate of ascent during capture, thus, is always too rapid for a rockfish to be able to resorb the excess gas in its swimbladder. It is this excess swimbladder gas which causes the barotrauma indicators described above.

Stress physiology in fish

When a fish perceives a stressor, or a threat to its homeostasis, a cascade of physiological responses to the threat occurs. This response to stress was first generalized by Hans Selye (1936) as the General Adaptation Syndrome (GAS). In the GAS there are three stages involving 1) an alarm reaction to a stimulus, 2) a stage of resistance where the organism tries to adapt and regain homeostasis, and 3) a stage of exhaustion where the

organism was unable to adapt and achieve homeostasis. Although the science of stress physiology has come a long ways since the GAS was first proposed, and many scientists find the concept flawed, it still provides a useful framework for discussing the concept of stress (Barton 1997). Today, we still categorize the physiological response to stress into three different levels (Barton 2002), although with much more detail. In the primary response, the neuroendocrine system secretes catecholamines from chromaffin cells and stimulates the hypothalamus-pituitary-interrenal (HPI) axis to release corticosteroids into the blood. In the secondary response, the released hormones cause changes in metabolic levels, osmoregulatory abilities, hematological features, and immunological function. The tertiary response results in changes to the whole fish, such as reduced growth and reproduction and performance capacities (Barton 2002, Schreck 2000).

During the primary stress response, sensory signals from the central nervous system stimulate chromaffin cells to release catecholamines into the blood and stimulate the hypothalamus to release corticotropin-releasing hormone into the anterior pituitary (Mazeaud et al. 1977). The anterior pituitary then secretes adrenocorticotropin (ACTH) into the blood, which acts on the interrenal cells of the head kidney. The interrenal cells, in response, synthesize and release corticosteroids into the blood for distribution to target tissues. In teleost fish, cortisol is the primary corticosteroid released (Donaldson 1981). Although catecholamines are released into the blood immediately after the stressor is perceived, there is a lag time of several minutes before the release of cortisol. Because of

this lag time, it is possible to obtain baseline levels of cortisol in fish, and compare it to levels several minutes after the stressor, to use as an indicator of the amount of stress experienced by the fish (Wendelaar Bonga 1997).

The secondary stress response involves a large number of physiological responses resulting from the increased levels of catecholamines and cortisol circulating in the blood. The effects of increased catecholamines include an increase in blood glucose levels (via glycogenolysis and gluconeogenesis and inhibition of glycolysis), an increase in plasma osmolarity (marine species), an increase in red blood cells released from the spleen, an increase in blood oxygen concentrations (due to an increase in pH), and an increase in oxygen uptake at the gills due to increased blood flow (Evans 1998, Mazeaud and Mazeaud 1981, Thomas 1990). Because the fish is likely hyperactive due to the stressor, and is actively breaking down glycogen into glucose (an anaerobic pathway), plasma lactate concentrations also increase (Wedemeyer and McLeay 1981). Increased levels of cortisol will maintain the hyperglycemia caused by an increase in catecholamines by stimulating protein catabolism and gluconeogenesis (Leach and Taylor 1980). In addition, increased cortisol causes a decrease in white blood cells, and a reduced immune response to infections (Schreck 1996). This secondary stress response is meant to mobilize energy reserves so the fish can respond to the perceived stressor and typically only lasts a few days after exposure to the stressor, after which cortisol levels

return to normal. If the stressor is chronic, however, the response will progress to the tertiary level.

In the tertiary stress response, the whole fish is affected. Chronically high levels of circulating cortisol have been linked to a decrease in disease resistance, reduced growth, reduced reproductive success, and a decrease in performance capacity (Mommsen et al. 1999, Schreck 2000, Wedemeyer and McLeay 1981). If the fish cannot adapt to the chronic stressor, then the stressor will be lethal as a result of the secondary and tertiary responses.

Many studies have researched the physiology of various short and long-term stressors in fish including crowding, handling, transport, temperature, and contaminant exposure (Barton 2002), however little work has been completed on how fish respond, physiologically, to the stress of pressure-related trauma or determined if fish can recover from such a severe stress.

Gene Expression and Microarrays

When an organism perceives a stressor, production of certain proteins may be upregulated while production of other proteins may be downregulated to deal with the stressor. Identifying different levels of gene expression is a tool that can be used to characterize how different proteins are regulated in response to different biological

stressors. With technology such as the cDNA microarray (Gracey and Cossins 2003), we can investigate which proteins may be produced in response to a stressor such as barotrauma at the transcript level. By measuring the amount of mRNA present from different genes, we can determine what types of proteins will likely be produced in response to the stressor. These proteins can also be used to assess recovery from the stressor.

In a cDNA microarray, complementary DNA (cDNA) sequences encoding different genes are spotted onto a glass slide. The slide can hold thousands of different genes, often called features. Next, mRNA is extracted from the target tissue and used to synthesize cDNA. Flourescent probes are attached to the sample cDNA (Gracey and Cossins 2003). To compare two different groups, different flourescent labels are attached to sample cDNA from each group. The sample cDNA is then hybridized to the cDNA microarray. Bound cDNA from the first group will flouresce one color (ie: red), while bound cDNA from the second group will flouresce a different color (ie: green). The amount of flourescence is directly related to the amount of bound cDNA, thus if the amount of mRNA expressed by a particular gene is higher in the first group than in the second group, the color is red. If the amount of mRNA expressed by a particular gene is about equal in both the groups, the color is yellow. The amount of flourescence for a particular gene from the two groups can be quantified into a gene expression value (Cheung et al. 1999).

In most cDNA microarrays, the features spotted on the glass slide have a known function. However, for many non-model organisms where few genes have been sequenced, custom cDNA microarrays are created. In a custom cDNA microarray, the cDNA features spotted onto the microarray are often sequences with an unknown function. The sequences are derived from a cDNA library created with mRNA from the non-model organism. Genes that show high levels of differential expression can be selected for sequencing and identified by homology in a Blast search of the GenBank database. Genes that are of interest can then be validated using quantitative real-time PCR.

Research objectives

In this project, we study the injuries that occur in rockfish during barotrauma and the potential for rockfish to recover from these injuries using a macroscopic to microscopic to molecular approach. Previous research on barotrauma in rockfish has only focused on the macroscopic injuries associated with barotrauma (Hannah et al. 2008a, Hannah et al. 2008b), the short-term recovery potential (Hannah and Matteson 2007, Jarvis and Lowe 2008), or the long-term delayed mortality and rate of swimbladder healing (Parker et al. 2006). Little work has been done to assess barotrauma injury or long-term recovery at the microscopic, physiological or molecular levels.

In the first two chapters of this project, we assess injury resulting from barotrauma in several different species of rockfish using both macroscopic indicators and tissue-level histologic analyses of the pseudobranch, gill, heart ventricle, head kidney, liver, and rete mirabile. For the first chapter, we use hyperbaric pressure chambers to assess barotrauma injury in three nearshore species of rockfish that underwent simulated decompression from 4.5 ATA (approximately 35 m depth). In the second chapter, we used hook and line sampling at sea to assess barotrauma injury in six different species of rockfish captured at depths ranging from 25 m to 144 m.

In the final two chapters of this project we assess the potential for recovery from barotrauma in black rockfish that have undergone simulated decompression from 4.5 ATA with subsequent recompression. We investigated the longer-term potential for recovery over a 31 day period using macroscopic indicators, tissue histology, blood plasma enzymes indicative of tissue injury, the blood plasma hormones cortisol and insulin-like growth factor I, blood plasma metabolites, and differential patterns in gene expression between treatment and control fish. The third chapter describes the physiology of recovery utilizing the results from macroscopic, tissue, and blood plasma analyses. The fourth chapter describes the creation of a rockfish-specific cDNA microarray to assess barotrauma injury and recovery at the molecular level.

By studying the response to barotrauma and the recovery from barotrauma at multiple levels, we can provide fishermen and fishery managers with a greater understanding of the potential for rockfish to recover from barotrauma.

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The differential response to decompression in three species of nearshore Pacific rockfish

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North American Journal of Fisheries Management

American Fisheries Society

5410 Grosvenor Lane, Bethesda, MD 20814

29 (5): 1479-1486

doi: 10.1577/M08-234.1

Introduction

The *Sebastes* genus (rockfishes) is an ecologically diverse group of temperate marine fish with over 60 species occurring in the Northeast Pacific Ocean (Love et al. 2002). Rockfish are important to both commercial and recreational fishing industries, but since the mid 1980s populations have been in decline (Love et al. 2002). As of 2008, seven species of rockfish in the Northeast Pacific have been designated as "depleted" by the Pacific Fisheries Management Council (PFMC 2008). Depleted rockfish populations will take many years to recover because rockfish are long lived and late maturing. Most species will live 40 to 100 years and will not mature until 5-10 years of age (Love et al. 2002). In addition, rockfish often occur in mixed-species assemblages, making it difficult for fishermen to target only a particular species. As a result, bycatch of non-target rockfish species is a common occurrence. In the commercial fishery, most rockfish are captured using trawls; longlines and other fixed gears are also used, but to a lesser extent. Rockfish captured as bycatch using these methods are often severely injured by the time they reach the surface due to abrasions from other fish and the long period in the net or on a hook. In the recreational fishery, rockfish are captured using hook and line. Management efforts in the recreational fishery currently include bag limits, size limits, gear restrictions, time/area closures and discard of species of concern (2009). Discard is necessary so depleted species will not be targeted, but often discard mortality of rockfish is high because as rockfish are captured and undergo a forced ascent, the pressure change

can cause barotrauma and complications due to barotrauma (excessive buoyancy and or shock) which prevent successful release.

Barotrauma is a condition that results from swimbladder gas expanding as fish are brought up from depth (forced decompression). According to Boyle's Law, as pressure decreases, gas expands exponentially. Many rockfish experience barotrauma because they are physoclists, which means they have a closed swimbladder (unlike physostomes such as salmonids, whose swimbladder is connected to their esophagus via a duct, allowing uptake/release of gas through the mouth). As a rockfish undergoes forced decompression, the expanding gas inside the swimbladder often leaks into the peritoneal and cranial cavities (Hannah et al. 2008b). The excess swimbladder gas can result in bloating, ruptured swimbladder, crushed organs, eversion of the esophagus, and exopthalmia as well as excessive buoyancy (Gotshall 1964, Hannah and Matteson 2007, Hannah et al. 2008a, Jarvis and Lowe 2008, Rummer and Bennett 2005). Excessive buoyancy makes it difficult for many rockfish species to submerge on their own. Discarded rockfish are often left floating on the surface where they succumb to predation by birds, and/or thermal shock.

Hannah et al.(2008a) investigated levels of submergence success of nearshore rockfish species when captured from different depths and found some species (blue *S. mystinus*, canary *S. pinniger*, widow *S. entomelas*) have difficulty submerging when captured at

depths exceeding 30 m. Recent research suggests recompression of rockfish using devices such as weighted cages and barbless weighted hooks may increase survival of discarded rockfish because gases recompress, external indicators of barotrauma disappear, and fish are often able to swim away (Hannah and Matteson 2007, Jarvis and Lowe 2008). However, little information on the tissue-level effects of barotrauma in rockfish is available. Parker et al. (2006) investigated acclimation rates to pressure changes and the healing of ruptured swimbladders in black rockfish *S. melanops* decompressed from four atmospheres absolute (ATA) (30 m). Most other studies (Hannah and Matteson 2007, Hannah et al. 2008a, Jarvis and Lowe 2008, Rogers et al. 2008) have focused only on macroscopic observations of decompressed rockfish with no investigation of potential tissue-level injury. In order to gain a better understanding of discard mortality in rockfish, it is important to determine how different species of rockfish respond to decompression both macroscopically and at the tissue level and how these injuries may relate to mortality.

In this study we investigated macroscopic and tissue-level responses to decompression from 4.5 ATA (35 m) in three species of nearshore rockfish commonly captured in the recreational fishery: black, blue, and yellowtail *S. flavidus* rockfish. Black rockfish are most common at depths less than 55 m, can live up to 50 years, and are often found schooling with other species in the water column (Love et al. 2002). Blue rockfish are most common at depths less than 90 m, can live up to 44 years, and also regularly

aggregate throughout the water column with other species (Love et al. 2002). Yellowtail rockfish are found a bit deeper, commonly at depths between 90 to 180 m, and are classified as both a nearshore and deep shelf species. Yellowtails can live to 64 years, and are also usually active in the water column during some part of the day (Love et al. 2002). Both yellowtail rockfish and black rockfish make rapid dives or ascents ranging more than 10 m in the water column (Parker et al. 2008, Pearcy 1992). Although none of these species are currently depleted, they are long-lived and late maturing, which could make them susceptible to more precipitous population declines in the future. By investigating both the macroscopic and tissue-level response of these species to decompression, we can provide fishery managers with more information on the potential for discard mortality of these species if they are discarded.

Methods

Fish collection

Approximately 19 of each adult black, blue, and yellowtail rockfish were collected off the coast of Newport, Oregon by hook and line from depths less than 15.2 m. Only rockfish with no or minimal indicators of barotrauma (swollen abdomen, air in the pharyngo-cleithral membrane) were utilized. We were unable to find large numbers of yellowtail rockfish and blue rockfish at the time of sample collection for these experiments, thus our sample size was limited to 19 for each species. Black rockfish sizes ranged from 33 to 41 cm in length, blue rockfish ranged from 29 to 41 cm in length, and

yellowtail rockfish ranged from 31 to 39 cm in length. These lengths fall within the size range for length at first maturity for each species (Love et al. 2002), thus we classified these fish as adults. Rockfish in these size ranges are commonly captured in the recreational fishery. Upon return to Newport, rockfish were immediately transferred into 2.4 m diameter flow-through tanks (106,000 L) at the Hatfield Marine Science Center where they were held until they resumed feeding and were neutrally buoyant (minimum 30 days). Cessation of feeding is a common response to stress (physical or perceived) and resumption of feeding can be an indicator that fish have recovered from the stress (Rice 1990). Neutral buoyancy ensures the swimbladder is functioning. Fish were held for a minimum of 30 days as a precaution to ensure recovery from any minor stressors as described in Parker et al. (2006). Other studies (McElderry 1979, Parker et al. 2006) have used this collection and holding technique on hundreds of black rockfish and no quantifiable effect from capture was seen in control fish in these studies.

Fish density was 10 - 15 fish per tank and flow rate was 12 - 15 L/min. Fish were fed a diet of thawed Atlantic silversides *Menidia menidia*, pink shrimp ***Pandalus jordani*** and California market squid ***Loligo opalescens*** three times a week and tanks were cleaned by siphoning debris daily. Dissolved oxygen (> 80% saturation), salinity (range: 34 – 37 ppt), and temperature (range: 9.4 - 14.4 °C) were also monitored daily.

Decompression Experiments

For each decompression experiment, we used a set of three hyperbaric pressure chambers that are described in Parker et al. (2006). Two pressure chambers served as treatment chambers and the third pressure chamber served as a control chamber. Three to four randomly chosen rockfish (of the same species) were placed in a single pressure chamber. In each experiment, one treatment chamber held one species, and the other treatment chamber held a different species. The pressure chambers were adjusted to 4.5 ATA (approximately 35 m depth) following a standard protocol as described in Parker et al. (2006). Fish were neutrally buoyant within approximately 7 - 10 days. Once neutrally buoyant, treatment fish were exposed to a simulated capture event by decreasing pressure to 1 ATA over a 90 sec period to induce decompression. This translates to a retrieval rate of 0.39 m/s, a rate close to what anglers would typically use. Control fish were slowly brought to surface pressure with a 10% pressure reduction every 2-3 hours over a period of three days. This controlled rate of ascent was determined by McElderry (1979) as slow enough for black rockfish to adjust with no physical damage. McElderry (1979) determined this rate of ascent by making small adjustments in pressure and observing the length of time it took for rockfish to become neutrally buoyant. We applied the same rate of ascent to blue rockfish and yellowtail rockfish after observing neutral buoyancy in each species prior to each pressure change.

This experiment was replicated three to four times for each species of treatment fish and two to three times for each species of control fish. For adult black rockfish and

yellowtail rockfish, a total of 12 treatment fish and seven control fish were sampled. For adult blue rockfish, a total of nine treatment fish and four control fish were sampled (Table 1). There are fewer blue rockfish due to a pump failure during one of the experiments. The pump failure caused a premature return to surface pressure in the chambers for both treatment and control blue rockfish. Because treatment rockfish were not fully acclimated to 4.5 ATA at the time, we were unable to use them for our study. Similarly, because control rockfish were not acclimated to 4.5 ATA and were decompressed too quickly we could not use them for our study.

Sample collection

Once at surface pressure, rockfish were immediately removed from the pressure chambers, and examined for external barotrauma indicators. External barotrauma indicators were recorded following Hannah et al. (2008a) and included: everted esophagus, tight abdomen, exophthalmia, ocular emphysema, and visible bulges or gas within the pharyngo-cleithral membrane (Figure 1). Emphysema refers to the abnormal accumulation of air in tissues; in this case air bubbles visible in the corneal region of the eye. The pharyngo-cleithral membrane refers to the membrane that is visible posterior to the gill filaments, bridging the area between the pharyngeal arch and the cleithrum (B. Hannah, Oregon Department of Fish and Wildlife, personal communication), referred to as the branchiostegal membrane in some studies (Hannah and Matteson 2007, Hannah et al. 2008a, Hannah et al. 2008b).

Rockfish were euthanized in an overdose of tricaine methane sulfonate (MS-222) and then dissected to sample the gill, pseudobranch, liver, head kidney, and heart ventricle. These tissues have been shown to be affected by decompression and/or gas bubble disease in other fish species (Beyer et al. 1976, D'Aoust and Smith 1974, Feathers and Knable 1983, Longbottom 2000). All fish were dissected within 20 minutes of euthanization. Tissue samples were fixed in Davidson's solution (Kent and Poppe 1998) at a ratio of no less than 1:10 (tissue:solution). During dissection, fish were also examined for any macroscopic signs of tissue injury such as ruptures in the swimbladder, unusual appearance of the liver or kidney, or hemorrhaging.

Sample Processing

Tissues were fixed for a minimum of 30 d and then sectioned to a thickness of 5-7 µm. Slides were stained with heamatoxylin and eosin Y. Slides were viewed with a Leica (model: DM LB, Leica Microsystems, Wetzlar, Germany) compound light microscope.

Statistical Analyses

In order to compare how different rockfish species respond to barotrauma, all comparisons are among treatment fish only. Due to small sample sizes Fisher's Exact Test was used to compare incidences of macroscopic barotrauma indicators both between tanks and between species. Count data was not transformed. Because multiple fish of a

single species were included in each tank during a replicate, a preliminary analysis was conducted for "tank effects" (greater variation than expected between tanks/experiments within each species under the binomial assumption). Fisher's Exact Test was also used to compare histological differences among species. Where differences were found, further Fisher's Exact tests were used to compare between species.

Results

Macroscopic Indicators

Control fish did not have any detectable injuries, while all treatment fish had at least minimal injuries. Because only treatment fish had observable injuries, we know injury was due to barotrauma and not due to handling or being held in the pressure chambers. No evidence of tank effects for the three species and seven barotrauma indicators was found ($P > 0.09$ for all 21 tests and $P > 0.5$ for all but four tests; df = 2 for yellowtail rockfish and blue rockfish, df = 3 for black rockfish). Therefore, between-species comparisons were conducted using the individual fish as the basic unit of analysis (i.e., ignoring tanks).

We only observed macroscopic barotrauma indicators in treatment fish (Figure 2). All barotrauma indicators were present in black rockfish and blue rockfish, while only air bubbles in the pharyngo-cleithral membrane and ruptured swimbladder were present in yellowtail rockfish. Yellowtail rockfish had lower incidences of inflated pharyngo-

cleithral membrane, everted esophagus, and ruptured swimbladder compared to black rockfish and blue rockfish ($P < 0.02$, df = 1). Yellowtail rockfish also had lower incidences of exophthalmia and ocular emphysema than black rockfish ($P = 0.0373$, df = 1).

We also observed a large color variation in the liver, in both treatment and control fish, ranging from a uniform creamy-yellow to reddish-orange to green. The reddish-orange and green colors either extended through the liver or were regionalized to the distal portion.

Histology

There was no detectable injury from barotrauma in the liver, head kidney, gill, or pseudobranch in either treatment or control fish. We did observe emphysema in the heart ventricle of treatment black rockfish and blue rockfish (Figure 3), which was characterized by distinct circular spaces representing bubbles in the cardiac muscle. Emphysema was not present in control rockfish and treatment yellowtail rockfish. The proportion of treatment black rockfish with emphysema in their heart ventricle was 0.58 (n = 12), and in blue rockfish it was 0.11 (n = 9). Black rockfish had higher levels of emphysema in the heart than blue rockfish ($P = 0.0272$, df = 1) or yellowtail rockfish ($P = 0.0007$, df = 1). Approximately 1 to 19 spherical gas bubbles per tissue section (area of tissue section: ~16 mm^2) were observed in the compact myocardium for rockfish with

emphysema (Figure 3C). Gas bubbles varied in size and on average were 0.015 mm^3 (±0.005). The proportion of gas bubble area to tissue area also varied, ranging from 0.2% to 6.3% of the tissue section.

Discussion

A variety of external barotrauma related changes occurred in treatment fish, but few internal injuries related to barotrauma were observed.

Yellowtail rockfish that were decompressed from 4.5 ATA did not have any external indication of barotrauma except for gas bubbles under the pharyngo-cleithral membrane. Yellowtail rockfish released gas bubbles from their pharyngo-cleithral membrane during decompression. Pearcy (1992) also made this observation and found the gas to be of the same composition as gas in the swimbladder. Because yellowtail rockfish are able to release excess gas in their swimbladder by way of their pharyngo-cleithral membrane, the trapped gas likely does not build up enough pressure to cause external indicators of barotrauma, such as everted esophagus and exophthalmia (Hannah et al. 2008b). Hannah et al. (2008a) showed that eversion of the esophagus and exophthalmia in rockfish is caused by gas escaping from an unruptured or ruptured swimbladder into the body cavity and moving in an anterio-dorsal direction.

All external barotrauma indicators were seen in black rockfish and blue rockfish. Hannah et al. (2008a) found similar proportions of external barotrauma indicators in field-captured black rockfish and blue rockfish when captured from depths between 30 and 39 m. A higher incidence of exophthalmia occurred in black rockfish and blue rockfish in our study, but this could be explained by the fact that the pressure change in our experiment was greater than experienced by fish in Hannah et al. (2008b). Although black rockfish and blue rockfish appear to respond similarly to decompression, studies on their ability to submerge after decompression (Hannah et al. 2008a), and on release behavior after recompression (Hannah and Matteson 2007), indicate they may not share the same ability to recover from decompression. In Hannah et al. (2008b) rockfish were captured from varying depths and tested for their ability to submerge on their own. Black rockfish (n = 73) captured from between 30 and 39 m depths were able to submerge 82% of the time, while blue rockfish (n = 25) captured from the same depths were only able to submerge 28% of the time. Differences are also evident between black rockfish and blue rockfish after release from a recompression cage (Hannah and Matteson 2007). Black rockfish showed significantly less behavioral impairment from barotrauma when captured between 12 and 39 m depths than blue rockfish.

Internally, the only macroscopic barotrauma injury we observed was swimbladder rupture. This was observed in most black rockfish and blue rockfish, and in a small percentage of yellowtail rockfish. Yellowtail rockfish likely have a low incidence of

swimbladder rupture because of their ability to off-gas, as described above. Parker et al. (2006) decompressed and recompressed 90 black rockfish from 4.0 ATA using hyperbaric pressure chambers and observed ruptured swimbladders in 100% of the fish, which is similar to the 83% ruptured swimbladders we found for black rockfish. The same study observed partial healing of the swimbladders by 21 days post-treatment, in 77% of the fish. Nichol and Chilton (2006) found Pacific cod *Gadus macrocephalus* with ruptured swimbladders were able to heal their swimbladders enough to hold gas within 24 hours, and Bellgraph et al. (2008) found juvenile rainbow trout *Oncorhynchus mykiss* could completely heal ruptured swimbladders after 14 days. This suggests that a ruptured swimbladder may not be an irreversible injury.

Macroscopically, the livers of control and treatment fish showed a variety of colors, ranging from a uniform creamy-yellow to reddish-orange to green. The reddening of the liver was likely due to post-mortem congestion and the green livers were due to bile congestion as a result of starvation (McGavin and Zachary 2007). Rockfish could not be fed for the 14-day duration of the experiments; thus all had distended gall bladders filled with dark green bile at the time of sampling. Regardless of the cause of color variation in the liver, it is important to note that no histological changes were correlated with these color variations. This emphasizes that caution should be taken when assigning tissue damage to the liver (e.g., hemorrhage) based solely on macroscopic observations.

The only tissue-level injury directly attributable to decompression was emphysema in the heart ventricle of treatment black rockfish and blue rockfish, concentrated in the compact myocardium. Longbottom (2000) also observed lesions in the heart ventricle of the Australasian snapper *Pagrus auratus* after capture from depths ranging between 10 – 35 m, and D'Aoust and Smith (1974) observed histological evidence of gas bubbles in the somatic muscle tissue of fingerling rainbow trout and coho salmon *Oncorhynchus kisutch* after being decompressed from 4.0 ATA. Whereas lesions representing entrapped bubbles have been found in many other organs in fishes with pressure related diseases (Beyer et al. 1976, D'Aoust and Smith 1974, Kulshrestha and Mandal 1982, Pauley and Nakatani 1967, Smith 1988, Speare 1998) we did not find these outside of the heart in our study. The lack of internal injury in the liver, gill, head kidney, and pseudobranch may be related to the relatively short period of time these internal organs were exposed to high gas pressure. Because gas expands exponentially as pressure decreases, most gas expansion in the swimbladder will occur within the final few meters of the surface. When a fish is brought to the surface, internal organs have been exposed to high gas pressures for just seconds, which is perhaps not enough time for widespread gas-related internal injury to occur. In other pressure related diseases, such as gas bubble disease, supersaturated water gradually supersaturates the fish over several hours to days. The extended exposure to supersaturating gas in gas bubble disease increases the likelihood of internal injury to tissues (Bouck 1980). Strauss (1979) described tissues as either "fast" or "slow" in their uptake of gas, with well perfused tissues being "fast" and poorly

perfused tissues being “slow.” The heart ventricle is a well perfused tissue and thus may develop emphysema more quickly than other tissues. Alternatively, excess gases are quickly eliminated following decompression (Speare 1998) and in soft tissues evidence of lesions from short-term gas bubbles may disappear.

It is unknown how emphysema in the heart ventricle tissue will affect fish performance in the field. Bouck (1980) suggests tissues with emphysema from gas bubble disease can become necrotic and infected, but this refers to emphysema that has developed from prolonged exposure to supersaturating conditions, not short-term exposure as in our study. Research on injured hearts from zebrafish *Danio rerio* indicate zebrafish have a remarkable capacity for cardiac regeneration (Lepilina et al. 2006) after major injury to the heart ventricle; it is possible this could apply to rockfish as well, and should be investigated.

Our study was removed from the natural environment and thus does not address additional issues rockfish face as a result of capture such as thermal shock, air exposure, and hooking or handling injury. However this study does provide insight into what happens in rockfish undergoing rapid decompression from a quantifiable depth. Fish that perform vertical migrations, such as yellowtail rockfish and black rockfish, are often not neutrally buoyant at their depth of capture; they often maintain negative buoyancy in order to reduce the effect of gas expansion as they swim higher in the water column

(Strand et al. 2005). Thus, it is difficult to quantify depth of capture and relate it to the severity of barotrauma in many species of rockfish without removing the fish to artificial conditions.

Our study is the first to examine barotrauma injury in rockfish from a macroscopic to microscopic level. Macroscopic injuries in black, blue, and yellowtail rockfish were similar to other published studies on rockfish (Hannah and Matteson 2007, Hannah et al. 2008a, Jarvis and Lowe 2008, Parker et al. 2006); however in addition to macroscopic injuries, we also found black rockfish and blue rockfish can develop emphysema in the heart ventricle. Surprisingly, no injury was found at the histological level in the head kidney, liver, gill, and pseudobranch, suggesting these tissues may be resilient to short term exposure of elevated gas pressure when decompressed from 4.5 ATA. Our research also indicates a species-level difference in the response to barotrauma in rockfish and that management of discard mortality in rockfish will depend on understanding the species-specific responses to decompression.

Acknowledgements

We thank Cliff Pereira for help with statistical analyses. We thank the personnel at the Oregon Department of Fish and Wildlife (ODFW), Newport, OR who assisted in fish collections and sample collections. We thank Polly Rankin (ODFW) who assisted and taught us fish collection, fish husbandry, and operation of the hyperbaric pressure

chambers. We also thank the reviewers for valuable comments. This research was funded by the Coastside Fishing Club (San Francisco, CA) and the Oregon Department of Fish and Wildlife (Newport, OR). Publication costs were funded by the Thomas G. Scott Grant through Oregon State University's Department of Fisheries and Wildlife.

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Table 1. Number of black, blue, and yellowtail rockfish used in decompression experiments. Treatment refers to rockfish that experienced rapid decompression from 4.5 ATA, and control refers to rockfish that experienced a controlled ascent to the surface over a three day period.

Species	Treatment	Control
Black	12	7
Blue	9	4
Yellowtail	12	7

A. B.

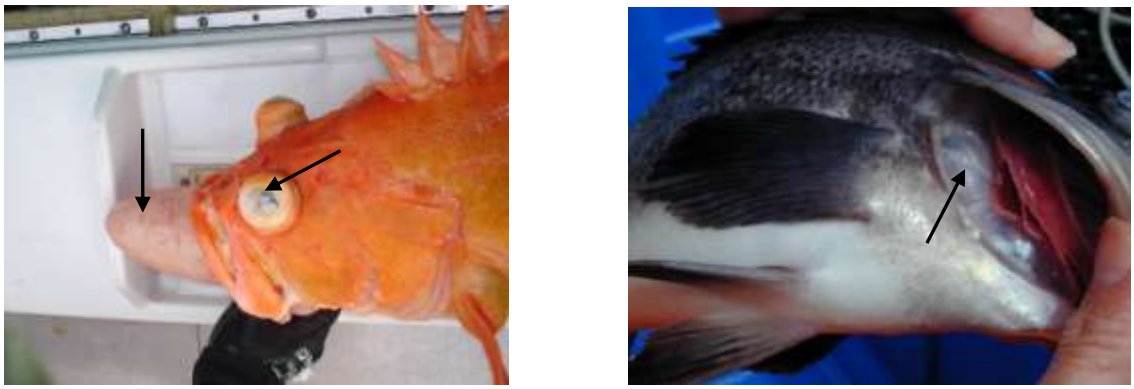


Figure 1. Barotrauma indicators in rockfish. A) Arrows indicate everted esophagus, exophthalmia, and ocular emphysema. B) Arrow indicates inflated pharyngo-cleithral membrane and air in the pharyngo-cleithral membrane (photo by Polly Rankin, Oregon Department of Fish and Wildlife).

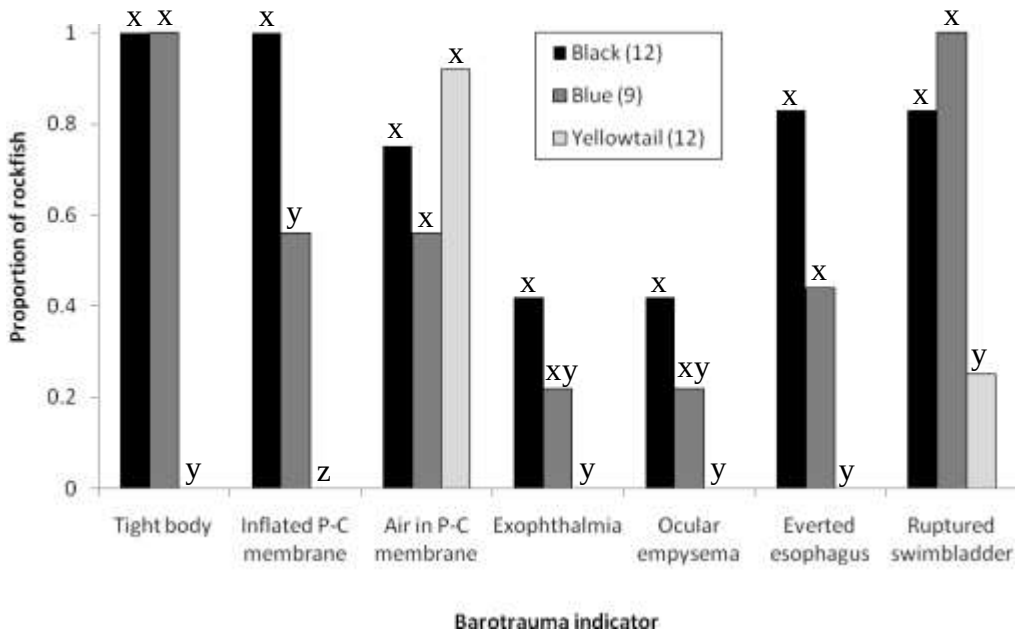
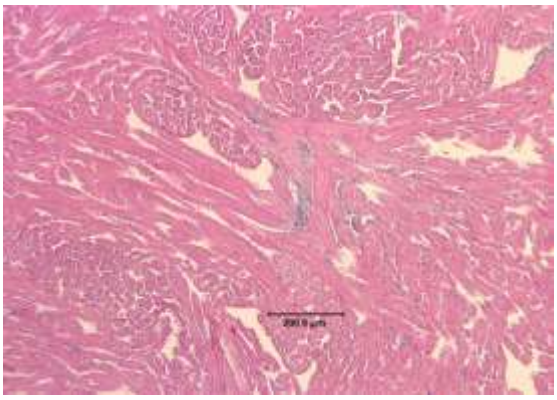


Figure 2. Proportion of black, blue, and yellowtail rockfish with macroscopic barotrauma indicators after decompression from 4.5 ATA. Different letters indicate evidence of a difference at the 0.05 level using Fisher's Exact Test. P-C membrane = pharyngo-cleithral membrane.

Figure 3. Histology of rockfish heart ventricles. A) Normal heart ventricle from a control fish. Magnification 100x. B) Heart ventricle from a treatment fish with emphysema (gas bubbles in the tissue). Arrows indicate individual gas bubbles. Magnification 100x. C) Lower magnification view of tissue section; note gas bubbles are located near the periphery of the tissue section. Magnification 25x.

A.



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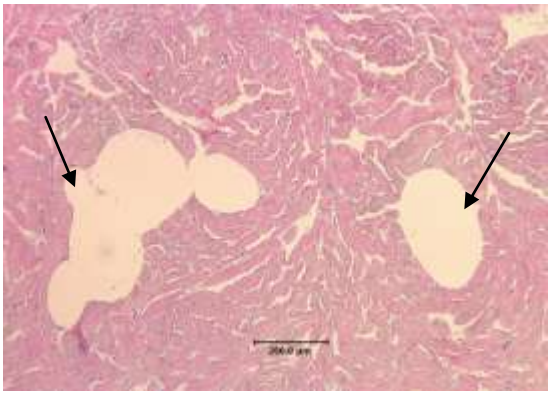

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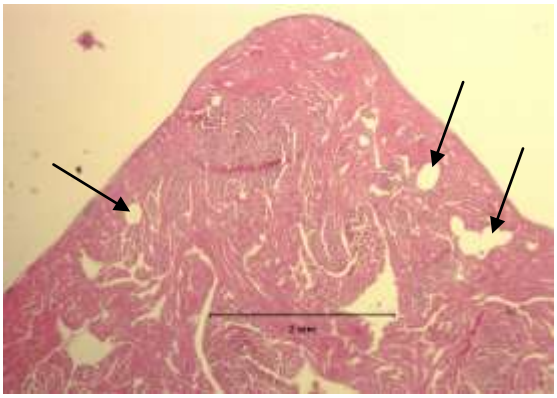

Figure 3.

The response to barotrauma in six species of Pacific rockfish and baseline cortisol and parasite conditions

Alena L. Pribyl, Michael L. Kent, Steven J. Parker, and Carl B. Schreck

Introduction

The rockfish genus, *Sebastes,* is very diverse with over 65 species occurring off the West coast of North America from Baja California to Alaska (Love et al. 2002). Rockfish species inhabit areas from nearshore to the continental slope, with both benthic and semi-pelagic life histories. Most rockfish species have an average lifespan of 40 to 100 years, and reach reproductive age between 5 to 15 years of age (Love et al. 2002).

Rockfish are central to the fishing economies of the west coast of North America. Rockfish are captured in both recreational and commercial fisheries (Parker et al. 2000), and because rockfish occur in mixed-species assemblages and often have similar feeding habits, targeting a given species is difficult and bycatch is a common problem. Seven species of rockfishes are currently classified as "depleted" by the Pacific Fishery Management Council (PFMC 2008). Because of long generation times, recovery of rockfish populations is expected to be slow. In recreational fisheries, rockfish are captured using hook and line. Management efforts in the recreational fishery currently include bag limits, size limits, gear restrictions, time/area closures and discard of species of concern (2009). Discard is necessary so depleted species will not be targeted, but often discard mortality of rockfish is high because as rockfish are captured and undergo a forced ascent, the pressure change can cause barotrauma and complications due to barotrauma (excessive buoyancy and or shock) which prevent successful release.

Barotrauma is a condition that results from swimbladder gas expanding as fish are brought up from depth (forced decompression). According to Boyle's Law, as pressure decreases, gas expands exponentially. Rockfish are physoclists (have a closed swimbladder), thus, as rockfish undergo forced decompression during capture, the expanding gas inside the swimbladder can leak into the peritoneal and cranial cavities (Hannah et al. 2008b). The excess swimbladder gas can result in bloating, ruptured swimbladder, crushed organs, eversion of the esophagus, exopthalmia, emphysema in the heart tissue, as well as excessive buoyancy (Gotshall 1964, Hannah and Matteson 2007, Hannah et al. 2008a, Jarvis and Lowe 2008, Pribyl et al. 2009, Rummer and Bennett 2005). Excessive buoyancy makes it difficult for many rockfish species to submerge on their own. Discarded rockfish are often left floating on the surface where they succumb to predation by birds, and/or thermal shock.

Research has been conducted to identify the external effects of barotrauma in different species of rockfish (Hannah and Matteson 2007, Hannah et al. 2008b, Jarvis and Lowe 2008) and their ability to submerge after capture (Hannah et al. 2008a), however little work has been done to systematically evaluate the effects of decompression on organs or tissues in rockfishes. The only study to investigate tissue-level effects of decompression in rockfish (Pribyl et al. 2009) decompressed black *Sebastes melanops*, blue *S. mystinus*, and yellowtail *S. flavidus* rockfish from 4.5 atmospheres absolute (ATA) using hyperbaric pressure chambers. The only injuries they found due to decompression was

emphysema or gas bubbles in the heart tissue of black rockfish and blue rockfish and ruptured swimbladders in all three species. They found no tissue-level injury in the liver, head kidney, gill, or pseudobranch. These findings should be confirmed in the field, and additional species that inhabit greater depths should be investigated.

In this study, we investigated the macroscopic and histological response to forced decompression is six species of Pacific rockfish captured from a variety of depths. In addition, we provide information on baseline conditions in wild rockfish such as baseline plasma cortisol levels, parasite presence and associated lesions in different tissues. Rockfish species included: black, blue, yellowtail, canary *S. pinniger*, quillback *S. maliger*, and yelloweye *S. ruberrimus* rockfish. Black rockfish and blue rockfish are common at depths less than 90 m and regularly aggregate throughout the water column. Yellowtail rockfish are common at depths between 90 and 180 m and are also active in the water column. Canary rockfish are common between 80 and 200 m depths and are usually found near the bottom. Quillback rockfish are also bottom dwellers and can occur from subtidal to 274 m. Yelloweye rockfish are most common between depths of 91 to 180 m and are found on or near the ocean bottom (Love et al 2002). All six species of rockfish are commonly captured in the recreational and commercial fisheries, and both canary rockfish and yelloweye rockfish are currently listed as depleted (PFMC 2008). By investigating the tissue-level response to decompression and baseline conditions in these

six species of rockfish we can provide information for future management of discard mortality in these species.

Methods

Sample collection

Rockfish were sampled immediately after capture by hook and line on chartered fishing vessels out of Newport, OR and Depoe Bay, OR between 2005 to 2007. Fifteen to 17 fish from each species were sampled (Table 1). Minimum lengths of each species were within the size range for length at first maturity for each species (Love et al. 2002). Depths of capture ranged from 20 to 194 m. Immediately upon capture rockfish were examined for external barotrauma indicators, total length measured, and if possible, sampled for blood by a caudal venipuncture using heparinized BD Vacutainers® (Becton, Dickinson and Company, Franklin Lakes, NJ) within five minutes of initial hooking. Blood samples could not be collected from every fish as not all charter boats had power available for the centrifuge. External barotrauma indicators were recorded and followed Hannah et al. (2008) and Pribyl et al (2009). These included: everted esophagus, swollen abdomen, inflated pharyngo-cleithral membrane, air bubbles in the pharyngo-cleithral membrane, exophthalmia, and ocular emphysema. Emphysema refers to the abnormal presence of gas in tissues (in this case, gas bubbles visible in the corneal region of the eye). Following blood sampling, rockfish were killed with a blow to the head and subsequent severing of the spinal column. Rockfish were then dissected and portions of

the heart ventricle, liver, head kidney, rete mirabile, and gill were removed within 20 minutes of capture and fixed in Davidson's solution at a ratio of no less than 1:10 (tissue : fixative). During the first year of sampling, not all tissues were collected, thus sample numbers by tissue vary. Recorded sample information included capture depth, length, sex, external barotrauma signs, and internal signs of injury such as ruptured swimbladder and hemorrhaging.

Sample processing

Blood samples were immediately placed on ice and centrifuged (1000 G x 6 min) within 15 minutes of collection; plasma was removed from the samples and stored on ice until return to the Hatfield Marine Science Center, where plasma was stored at -80 ºC until analysis. Plasma samples were later analyzed for cortisol using a radioimmunoassay following the protocol in Redding et al. (1984).

Tissues were fixed for a minimum of 30 d, processed into paraffin blocks, and then sectioned to a thickness of 5 – 7 um. Slides were stained with hematoxylin and eosin Y. In a few cases, additional slides were stained with Fite's acid fast stain. Slides were viewed with a Leica compound light microscope (Model DM LB; Leica Microsystems, Wetzlar, Germany) at magnifications between 100x to 400x. All images were obtained with SPOT Advanced imaging software (Diagnostic Instruments, Inc, Starling Heights, MI).

Statistical analyses

For macroscopic barotrauma indicators, we focused on four presence/absence indicators associated with more severe barotrauma: everted esophagus, exophthalmia, ocular emphysema, and ruptured swimbladder. We used Fisher's exact test to compare proportions of macroscopic barotrauma and histologic barotrauma injuries among species. When there were differences among species, we used further Fisher exact tests with a correction for false discovery rates (Waite and Campbell 2009) to determine where the differences were. We calculated 95% confidence intervals using Wilson's method (Wilson 1927), which has been demonstrated to work well with small sample sizes (Agresti and Coull 1998). We used Mann-Whitney tests for each species to determine if depth of capture was different between fish with and without each barotrauma injury. We used Fisher's exact test to compare proportions of each barotrauma injury by sex. We did not test for differences due to length because length varied linearly with depth of capture.

For cortisol data we used both a non-parametric Kruskal-Wallis test and a one-way ANOVA to compare cortisol levels among species. For the presence/absence of rete mirabile inflammation and parasites, we used Fisher's exact test to compare proportions among species with a correction for false discovery rates. All analyses were conducted on SPSS v.17.0.

Results

Macroscopic indicators

Analysis of macroscopic barotrauma indicators showed yellowtail rockfish and quillback rockfish were less affected by barotrauma indicators than black, blue, canary and yelloweye rockfish (Figure 1, Table 2). We did not observe everted esophagus, exophthalmia, ocular emphysema, or ruptured swimbladder in yellowtail rockfish. Mann-Whitney tests comparing the depth of capture between the presence/absence of barotrauma indicators within each species showed exophthalmia was associated with greater depths of capture in quillback rockfish; ocular emphysema was associated with grater depths of capture in quillback, canary, and yelloweye rockfish; and ruptured swimbladder was associated with lesser depths of capture in black rockfish (Table 3). Sex did not appear to affect the response to barotrauma (Fisher's exact test; $P > 0.20$ for all tests).

Histology

We observed emphysema (gas bubbles) in the heart ventricle (Figure 3B), emboli in the rete mirabile (Figure 3D), and emboli in the vessels of the head kidney (Figure 3F) as a result of barotrauma. No injury from barotrauma was observed in the liver or gill.

We observed low proportions of emphysema in the compact myocardium of the heart ventricle in all species of rockfish except for yellowtail rockfish (Figure 2, Table 4). We failed to detect a difference among species (Fisher's exact test; df = 1; $P > 0.05$ for all 15 tests when false discovery rate correction was applied). We also failed to detect a difference between the presence of emphysema in the heart ventricle and depth of capture in any of the species (Mann-Whitney test; $P > 0.1$ for all 6 tests). There was no evidence that the four macroscopic barotrauma indicators or sex affected the presence or absence of emphysema in the heart ventricle within each species (Fisher's exact test; df = 1; $P > 0.07$ for all tests). When we combined data from all species, however, we did find the presence of emphysema in the heart ventricle was related to the presence of the barotrauma indicator, everted esophagus (Table 5).

We observed emboli in the rete mirabile in all species of rockfish (Figure 2, Table 4). Emboli were isolated and on average, we found 1 to 5 emboli per cross section when emboli were present. We failed to detect a difference among species (Fisher's exact test; df = 1; $P > 0.05$ for all 15 tests). We also failed to detect a difference between the presence of emboli in the rete mirabile and depth of capture in any of the species (Mann-Whitney test; $P > 0.3$ for all 6 tests). There was no evidence that the four macroscopic barotrauma indicators or sex affected the presence or absence of emboli in the rete mirabile within each species (Fisher's exact test; df = 1; $P > 0.1$ for all tests) or when data from all species was combined (Table 5).

Emboli were also present in a few fish in the blood vessels of the head kidney in all species (Figure 2, Table 4). Again, emboli were isolated and on average we only found 1-2 emboli per cross section when emboli were present. We failed to detect a difference among species for the proportion of fish with emboli in the head kidney (Fisher's exact test; df = 5, $P = 0.269$). We also failed to detect a difference between the presence of emboli in the head kidney and depth of capture in any of the species (Mann-Whitney test; $P > 0.2$ for all 6 tests). There was no evidence that the four macroscopic barotrauma indicators or sex affected the presence or absence of emboli in the head kidney within each species (Fisher's exact test; df = 1; $P > 0.08$ for all tests) or when data from all species was combined (Table 5).

Tissue injuries not related to barotrauma included chronic inflammation in the rete mirabile and parasite infections in the heart ventricle, head kidney, liver, and gill. Chronic inflammation in the rete mirabile was characterized by large aggregations of macrophages and lymphocytes throughout the tissue, often obliterating capillaries (Figure 4A). Inflammation was primarily located in the capillary portion of the rete mirabile although in a couple cases it was also present in the gas gland. Yellowtail rockfish had higher proportions of rete mirabile inflammation than any other species, and black rockfish had the next highest proportions of inflammation (Table 6). Blue, quillback, canary, and yelloweye rockfish had no or very low proportions of inflammation.

Several parasites and lesions associated with parasitic or bacterial infection were observed in rockfish tissues. Blood flukes, both adults and eggs, were observed in the heart ventricle of all species of rockfish (Figure 4B, Table 6), although they were present in the highest proportions in blue and quillback rockfish. *Ichthyophonous* sp. infections were present in low proportions in the head kidney of black, quillback, and yellowtail rockfish and in the liver of black rockfish and yellowtail rockfish (Figure 4C, Table 6). Granulomas were present in the head kidney of black, canary, quillback, and yelloweye rockfish and in the liver of quillback rockfish and yelloweye rockfish (Figure 4D, Table 6). No bacteria suggestive of *Mycobacterium* sp. were observed in these granulomas when stained with an acid fast stain. Yelloweye rockfish had the highest proportion of granulomas in the head kidney (Table 6). Large, cartilaginous nodules were present in low proportions in the head kidney of all species of rockfish (Table 6). These nodules represent cartilaginous metaplasia as described in Heidel et al. (2002). Infection by a myxozoan was observed in the liver of black, blue, yellowtail and yelloweye rockfish (Figure 4E, Table 6). These infections consisted of coelozooic plasmodia with developed spores in the lumen of bile ducts. Spores had polar capsules at the opposing ends, and were consistent in morphology with *Zschokkella ilishae*. They were not associated with significant pathological changes. Small, larval nematodes were present in the livers of a small number of canary, quillback and yelloweye rockfish (Figure 4F, Table 6). One large anisakine nematode (L3 stage) was observed in the liver of a canary rockfish and

one blood fluke was observed in the bile duct of a black rockfish. A small number of rockfish had areas of focal inflammation in the liver, but these areas did not appear to be related to parasite infection. In the gill, we found four different infections: epitheliocystis was present in all species of rockfish (Figure 5A, Table 6), a monogenean parasite from the family Microcotylidae (likely *Microcotyle sebastis*) was present in black, blue, quillback and yellowtail rockfish (Figure 5B, Table 6), blood fluke eggs were present in all species of rockfish (Figure 5C, Table 6), and "cysts of unknown etiology" were present in three rockfish (one quillback and two yellowtail; Figure 5D).

Cortisol

Cortisol values were highly variable in all species and ranged from 0 (non-quantifiable) to 54.1 ng/ml (Figure 6). We failed to detect a difference in unstressed cortisol means between species (Kruskal-Wallis; df = 5; $P = 0.281$; ANOVA; df = 5, F = 0.642, $P = 0.669$).

Discussion

Tissue-level responses to barotrauma included embolism and emphysema in several tissues in all species; however, the macroscopic-level response to barotrauma tended to be species-specific. Presence of parasite infections in rockfish also showed species-specific differences, likely because of the incredible diversity of life histories of individual rockfish species.

The macroscopic barotrauma indicators we observed were species-specific, which is a similar finding as several other studies on rockfish (Hannah and Matteson 2007, Hannah et al. 2008a, Jarvis and Lowe 2008, Pribyl et al. 2009). Similar to Hannah and Matteson (2007) and Jarvis and Lowe (2008), we also found yellowtail and quillback rockfish to have few barotrauma indicators, if any. This is because both yellowtail rockfish and quillback rockfish are able to release excess gas by way of their pharyngo-cleithral membrane (Hannah et al. 2008a, Pearcy 1992, Pribyl et al. 2009). We also found that for several species, depth of capture played an important role in determining the presence and/or absence of a particular barotrauma indicator, although the barotrauma indicator varied by species. Jarvis and Lowe (2008) also found increasing depths of capture significantly affected the number of barotrauma indicators in rockfish. Decompression studies in other fish species such as red snapper *Lutjanus campechanus* (Gitschlag and Renaud 1994, Rummer and Bennett 2005), largemouthmouth bass *Micropterus salmoides* (Feathers and Knable 1983), Australasian snapper *Pagrus auratus* (Longbottom 2000), and West Australian dhufish *Glaucosoma hebraicum* (St John and Syers 2005), also show an increase in the effects of barotrauma with increasing depths of capture. Depth of capture was also shown to increase behavioral impairment in black, blue and yelloweye rockfish when released from a cage after capture and recompression (Hannah and Matteson 2007). Contrary to these studies though, we observed black rockfish were more likely to have a ruptured swimbladder when captured from shallower depths. One

possibility is that these black rockfish were neutrally buoyant at greater depths but swam higher into the water column after the fishing lure. Because black rockfish are known to undergo vertical migrations (Parker et al. 2008), we cannot assume our depth of capture was the depth at which the black rockfish were neutrally buoyant. Another possibility is that because the amount of gas expansion increases exponentially as pressure decreases, rockfish that are neutrally buoyant at shallow depths may experience a greater amount of gas expansion as they approach the surface.

At the tissue level we found more injuries due to barotrauma than Pribyl et al. (2009) did in black, blue and yellowtail rockfish decompressed from 35 m. As in Pribyl et al (2009), we observed emphysema in the heart ventricle, but in addition we observed emboli in the head kidney and rete mirabile, which have not been previously described in rockfish. Emphysema in the heart ventricle occurred in all species except for yellowtail rockfish, which included benthic as well as semi-pelagic species. This condition is likely a result of high gas pressures in the visceral and pericardium cavities as a result of ruptured or leaking swimbladders (Hannah et al. 2008). The presence of emphysema in most rockfish species suggests the heart ventricle, being a highly perfused organ, is more sensitive to excessive gas exposure than other less well-perfused organs (Pribyl et al. 2009, Speare 1998). We speculate that yellowtail rockfish likely did not have emphysema in the heart because gas pressure near the heart did not build up to the same levels as in the other species. We also found emboli in the head kidney and rete mirabile

in all species of rockfish. Pribyl et al. (2009) did not find any injury in the head kidney in rockfish that were decompressed from 35 m, however most of the rockfish in this study were sampled from depths much greater than this.

The rete mirabile is a gas-concentrating organ inside the swimbladder consisting of venous and arterial capillaries side by side. Because it is the gas in the swimbladder that expands as a fish is brought to the surface, gas pressure in the swimbladder increases dramatically, exposing the rete mirabile to high gas pressures as well. Thus, it is not surprising we found emboli in the rete mirabile and that the emboli were related to the presence of a ruptured swimbladder. The presence of emboli in the rete mirabile and head kidney could block blood flow and it is also possible that emboli could coalesce to block blood flow in progressively larger vessels (Bouck 1980), if left untreated. Little work has been done at the tissue level in fish with regard to decompression, and this is the first work to show emboli at the histologic level in the rete mirabile and head kidney. Emboli have been observed with histology in the heart (Edsall and Smith 1991, Smith 1988), gill (Edsall and Smith 1991, Smith 1988, Speare 1998), and eye (Smith 1988, Speare 1998) of fish suffering from gas bubble disease; however, in gas bubble disease fish are exposed to supersaturating concentrations of gas originating from the water, not from high gas concentrations originating within themselves. Because emboli from decompression are formed rapidly, if rockfish are recompressed quickly after capture so that gases recompress, emboli should disappear and likely not cause any long-term injury.

We found no species-specific differences in the presence of emphysema in the heart ventricle and emboli in the rete mirabile and head kidney. Because of small sample sizes it is unclear if there is truly no species-level effect or if the sample size was not large enough to allow us to detect a difference among species.

It is noteworthy that we found no injury due to barotrauma in the liver and gill in all species of rockfish examined. It appears that barotrauma may not affect these tissues in rockfish, and perhaps this is due to the short period of time these internal organs are exposed to high gas pressure (Pribyl et al. 2009).

In-situ tissue conditions of chronic inflammation in the rete mirabile and parasite infection by blood flukes in the heart yielded some interesting differences among rockfish species. Chronic inflammation results when inflammatory stimuli are not eliminated from an acute inflammatory response (Roberts 2001). This response can be triggered by a wide range of factors including diet, or infection with bacteria, fungi, or parasites (Secombes 1996). We did not find any evidence of bacterial, fungal, or parasitic infection in the rete mirabile, leading us to believe some other mechanism is responsible for the inflammation. An interesting coincidence is that the two species with the greatest proportion of inflammation in the rete mirabile, black rockfish and yellowtail rockfish, are also both semi-pelagic and are known to make rapid dives or ascents ranging more than 10 m in the water column (Parker et al. 2008, Pearcy 1992). Although not tested, it

may be possible that continual changes in gas pressure caused by these dives may contribute to the inflammation. We also observed very high proportions of quillback rockfish and blue rockfish infected with adult blood flukes in the heart, while all other species had low proportions of infection. It is unclear why these two species should have such high rates of infection compared to other species. Two blood fluke species, *Aprocotyle macfarlani* and *Psettarium sebstodorum* (family Sanguinicolidae) have been described in rockfishes from the Pacific (e.g., black, yellowtail, canary and quillback rockfish) (Holmes 1971, Love and Moser 1983), and thus the flukes described here are likely one of these species.

We also observed low levels of infection by several parasites in the head kidney, liver and gill in all species of rockfish. *Ichthyophonous* sp. were present in the head kidney and/or liver of black, canary, quillback and yellowtail rockfish and is a common pathogenic fungus that infects freshwater and marine fishes (McVicar 1999). It occurs in a high prevalence in certain rockfish species in the Pacific Northwest (Kent et al. 2001). Criscione et al. (2002) demonstrated that the *Icthyophonous* spp. present in rockfish is likely a different species from *Ichthyophonus hoferi* which is present in Pacific herring *Clupea pallasi* and Chinook salmon *Oncorhynchus tshawytscha*. Myxozoans, belonging to the genus *Zschokkella,* were present in the bile ducts of the livers in black, blue, yelloweye and yellowtail rockfish. *Zschokkella ilishae* has been previously described in the gall bladder of *Sebastes* spp. (Love and Moser 1983, McDonald 1995, Stanley et al.

1992). This genus is characterized by the presence of polar capsules located at opposing ends of their spore, with the capsules located in different planes. Typical of coelozoic myxozoans, they were confined to the lumen of bile ducts and not associated with significant histopathological changes. Eggs of blood flukes were present in the gills of all species of rockfish. There are many reports of heavy infections by blood fluke eggs in the gills causing high mortality and morbidity (Ogawa and Fukudome 1994, Paperna 1995), but infections seen here were light (Love and Moser 1983). Monogeneans, likely of the species *Microcotyle sebastis,* were also found in the gill of black, blue, quillback and yellowtail rockfish. *M. sebastis* has been reported on the gill filaments of 35 different species of rockfish by Love et al. (2002) who suggests that most, if not all species of rockfish in the NE Pacific, are infected with this parasite. We also found three rockfish (1 quillback and 2 yellowtail) with cysts of unknown etiology in the gills. Maclean et al. (1987) describes these cysts and presumes the cysts are remnants of degraded parasites, although thorough investigation found no identifiable organisms within the nodules. These cysts have been found in the gills, liver and kidney in both marine and freshwater fish (Maclean et al. 1987).

Lesions, possibly associated with bacterial infections, were also observed in the head kidney, liver and gill of rockfish. Granulomas, several of which were *Mycobacterium*-like, were in the head kidney and liver of rockfish. We were not able to visualize acid-fast bacilli indicative of *Mycobacterium* in these granulomas; however, it is well

recognized that it is often difficult to visualize mycobacteria in acid fast stained tissues, even when the presence of infection has been confirmed by either culture or molecular methods (Canale et al. 2000, Kaattari et al. 2005). *Mycobacterium* infections have been found in several rockfish species (Kent et al. 2001), and as here it was difficult to detect the bacterium in acid fast sections. Whipps et al. (2003) showed that the mycobacterium discussed by Kent et al. (2001) was related to *Mycobacterium triplex* using rDNA sequences obtained directly from tissues. Cartilaginous metaplasia was present in the head kidney of all rockfish species. Heidel et al. (2002) described this condition in several species of rockfish and concluded this is a common lesion in the spleen and kidney of some rockfish species. Epitheliocystis was present in the gill of all species of rockfish. Epitheliocystis is a common condition infecting marine and freshwater fish species and is thought to be caused by bacteria from the order Chlamydiales (Nowak and LaPatra 2006). It only causes morbidity in heavy infections though, and all infections seen here were light.

Cortisol is the primary corticosteroid released in teleost fish (Donaldson 1981). By knowing unstressed levels of cortisol in individual rockfish species, future studies can compare cortisol levels from these species of rockfish with the baseline levels to determine if cortisol output is above normal (Wendelaar Bonga 1997). Because cortisol has a lag time before it is released (Barton 2002) and rockfish in our study were sampled for blood within five minutes of initial hooking, it is unlikely cortisol levels will have had

time to respond to the capture stress. Although we cannot be certain rockfish were unstressed at the time of capture (ocean conditions may have differed between trips) we believe our samples are representative of baseline cortisol levels of rockfish in the wild.

In this study we investigated how different tissues respond to barotrauma and gathered more information on the in-situ conditions of different tissues in wild rockfish. We found highly perfused tissues exposed to high gas pressures such as the heart ventricle, rete mirabile and head kidney can be injured from decompression, while the liver and gill showed no sign of injury. Except for the heart ventricle in yellowtail rockfish, no rockfish species was completely immune to the effects of barotrauma at the tissue level. Recompression studies do seem to indicate, however, that these injuries can be reversible if rockfish are immediately recompressed following capture (Pribyl et al. 2010, *in preparation-b*). In-situ conditions of different tissues such as the rete mirabile and heart ventricle did show species-specific effects, however parasite presence in other tissues did not appear to be species-specific.

Acknowledgements

We thank the charter boats *Misty*, *Miss Raven*, *Sampson*, *Tacklebuster*, and *Ilwaco Indian* for allowing us free passage to sample fish from their boats. We also thank Cliff Pereira and John Vansickle for assistance with statistics. This research was funded by the Coastside Fishing Club (San Francisco, CA), the Oregon Department of Fish and

Wildlife (Newport, OR), the Mamie Markham Research Award (Hatfield Marine Science Center), and Oregon State University's Department of Fisheries and Wildlife.

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Table 1. Number of fish sampled (N) in the field for each species, average length, average depth of capture (DOC), and sex ratio of rockfish used in the study. Ranges for length and DOC are in parentheses. Sex ratio = (# females) / (# males).

Species	**N**	**Total length (cm)**	**DOC (m)**	**Sex ratio**
Black	16	41.5 (39 – 47)	37.1 (26 – 46)	1.29
Blue	16	35.2 (34 – 42)	36.1 (20 – 48)	All female
Quillback	16	39.5 (35 – 46)	45.5 (29 – 77)	1.00
Yellowtail	15	39.0 (33 – 52)	56.0 (31 – 145)	2.75
Canary	17	38.1 (30 – 56)	62.2 (24 – 146)	3.25
Yelloweye	17	51.6 (31 – 62)	116.7 (37 – 194)	0.70

Table 2. Proportion of macroscopic barotrauma indicators in each species of rockfish. N = sample size. 95% CI = 95% confidence intervals calculated using Wilson's method. Superscript letters represent significant differences between species ($P < 0.05$; Fisher's exact test).

Indicator	Species	N	Proportion	95% CI
Everted esophagus	Black[a]	16	0.88	0.64 – 0.97
	Blue[a]	16	0.81	0.57 – 0.93
	Quillback[b]	16	0.06	0.01 – 0.28
	Yellowtail[b]	15	0.00	0.00 – 0.20
	Canary[a]	17	0.77	0.53 – 0.91
	Yelloweye[a]	17	0.94	0.73 – 0.99
Exopthalmia	Black[a]	16	0.19	0.07 – 0.43
	Blue[a]	16	0.06	0.01 – 0.28
	Quillback[a]	16	0.13	0.04 – 0.36
	Yellowtail[a]	15	0.00	0.00 – 0.20
	Canary[b]	17	0.71	0.47 – 0.87
	Yelloweye[b]	17	0.65	0.41 – 0.83
Ocular emphysema	Black[a]	16	0.00	0.00 – 0.19
	Blue[ab]	16	0.06	0.01 – 0.28
	Quillback[abc]	16	0.13	0.04 – 0.36
	Yellowtail[a]	15	0.00	0.00 – 0.20
	Canary[c]	17	0.47	0.26 – 0.69
	Yelloweye[bc]	17	0.41	0.22 – 0.64
Ruptured swimbladder	Black[a]	16	0.63	0.39 – 0.82
	Blue[ab]	16	0.44	0.23 – 0.67
	Quillback[b]	16	0.06	0.01 – 0.28
	Yellowtail[bc]	15	0.00	0.00 – 0.20
	Canary[abc]	17	0.29	0.13 – 0.53
	Yelloweye[abc]	17	0.24	0.10 – 0.47

Table 3. Summary of Mann-Whitney test results for determining if depth of capture differed by presence/absence of macroscopic barotrauma indicators. U = Mann-Whitney U statistic. Z = Z statistic from normal distribution.

Indicator	**Species**	**U**	**Z**	**df**	***P***
Everted esophagus	Black	8.0	-0.971	1	0.332
	Blue	11.5	-1.095	1	0.274
	Quillback	3.0	-0.987	1	0.324
	Canary	20.5	-6.25	1	0.532
	Yelloweye	4.0	-0.818	1	0.413
Exophthalmia	Black	17.0	-0.343	1	0.732
	Blue	1.5	-1.324	1	0.185
	Quillback*	1.0	-2.087	1	0.037*
	Canary	19.5	-1.110	1	0.267
	Yelloweye	20.5	-1.259	1	0.208
Ocular emphysema	Blue	1.5	-1.324	1	0.185
	Quillback*	1.0	-2.087	1	0.037*
	Canary *	16.0	-1.930	1	0.054*
	Yelloweye*	9.0	-2.542	1	0.011*
Ruptured swimbladder	Black*	11.0	-2.10	1	0.036*
	Blue	19.5	-1.292	1	0.196
	Quillback	5.0	-0.548	1	0.583
	Canary	17.0	-1.375	1	0.169
	Yelloweye	12.0	-1.588	1	0.112

Table 4. Proportion of histologic barotrauma indicators in each species of rockfish. N = sample size. 95% CI = 95% confidence intervals calculated using Wilson's method.

Indicator	Species	N	Proportion	95% CI
Heart ventricle emphysema	Black	16	0.13	0.04 – 0.36
	Blue	16	0.19	0.07 – 0.43
	Quillback	16	0.13	0.04 – 0.36
	Yellowtail	15	0.00	0.00 – 0.20
	Canary	17	0.06	0.01 – 0.27
	Yelloweye	13	0.39	0.18 – 0.65
Rete mirabile emboli	Black	15	0.67	0.42 – 0.85
	Blue	12	0.67	0.39 – 0.86
	Quillback	12	0.33	0.14 – 0.61
	Yellowtail	14	0.57	0.33 – 0.79
	Canary	12	0.33	0.14 – 0.61
	Yelloweye	12	0.25	0.09 – 0.53
Head kidney emboli	Black	16	0.13	0.04 – 0.36
	Blue	15	0.07	0.01 – 0.30
	Quillback	16	0.39	0.19 – 0.61
	Yellowtail	15	0.33	0.15 – 0.58
	Canary	17	0.29	0.13 – 0.53
	Yelloweye	12	0.25	0.09 – 0.53

Table 5. Summary of Fisher exact tests to determine if macroscopic barotrauma indicators affect the presence of histologic barotrauma indicators. Y/N indicates if a macroscopic barotrauma indicator was present. N = number of fish with or without the macroscopic barotrauma indicator. Proportion = the proportion of (N) fish with the histologic barotrauma indicator. *P* = the p-value from the Fisher's exact test.

Macroscopic Indicator	**Y/N**	**N**	**Histologic Indicator**	**Proportion w/ histologic indicator**	***P***
Everted esophagus*	Y	53	Heart emphysema	0.21	
	N	40		0.05	0.036*
Exophthalmia	Y	26		0.19	
	N	67		0.12	0.505
Ocular emphysema	Y	16		0.19	
	N	77		0.13	0.691
Ruptured swimbladder	Y	27		0.19	
	N	66		0.12	0.512
Everted esophagus	Y	41	Rete mirabile emboli	0.54	
	N	34		0.44	0.489
Exophthalmia	Y	21		0.38	
	N	54		0.54	0.305
Ocular emphysema	Y	14		0.29	
	N	61		0.54	0.137
Ruptured swimbladder	Y	22		0.50	
	N	53		0.49	1.000
Everted esophagus	Y	51	Head kidney emboli	0.18	
	N	40		0.33	0.139
Exophthalmia	Y	25		0.24	
	N	66		0.24	1.000
Ocular emphysema	Y	16		0.25	
	N	75		0.24	1.000
Ruptured swimbladder	Y	27		0.19	
	N	64		0.27	0.593

Table 6. Proportion of tissue injury not related to barotrauma in each species of rockfish. Superscript letters represent significant differences between species ($P < 0.05$; Fisher's exact test). RM = rete mirabile, Inflam = inflammation, BF = blood fluke, Ich = *Ichthyophonus* spp., CN = cartilaginous nodule, Myx = Myxozoan parasite, LN = larval nematodes, Epi = epitheliocystis, Mono = Monogenean parasite

	RM	**Heart**	**Head kidney**			**Liver**				**Gill**		
Species	Inflam	BF	Ich	Granuloma	CN	Ich	Granuloma	Myx	LN	Epi	Mono	BF
Black	0.50[b]	0.31[b]	0.06	0.13	0.19	0.06	0.00	0.25	0.00	0.13	0.06	0.06
Blue	0.00[c]	0.94[a]	0.00	0.00	0.33	0.00	0.00	0.13	0.00	0.20	0.20	0.07
Canary	0.08[c]	0.24[b]	0.06	0.12	0.19	0.00	0.00	0.00	0.06	0.24	0.00	0.06
Quillback	0.00[c]	0.94[a]	0.13	0.19	0.19	0.00	0.13	0.00	0.06	0.13	0.06	0.25
Yelloweye	0.08[c]	0.08[b]	0.00	0.38	0.50	0.00	0.15	0.08	0.15	0.23	0.00	0.08
Yellowtail	0.93[a]	0.27[b]	0.27	0.00	0.13	0.07	0.00	0.20	0.00	0.07	0.07	0.29

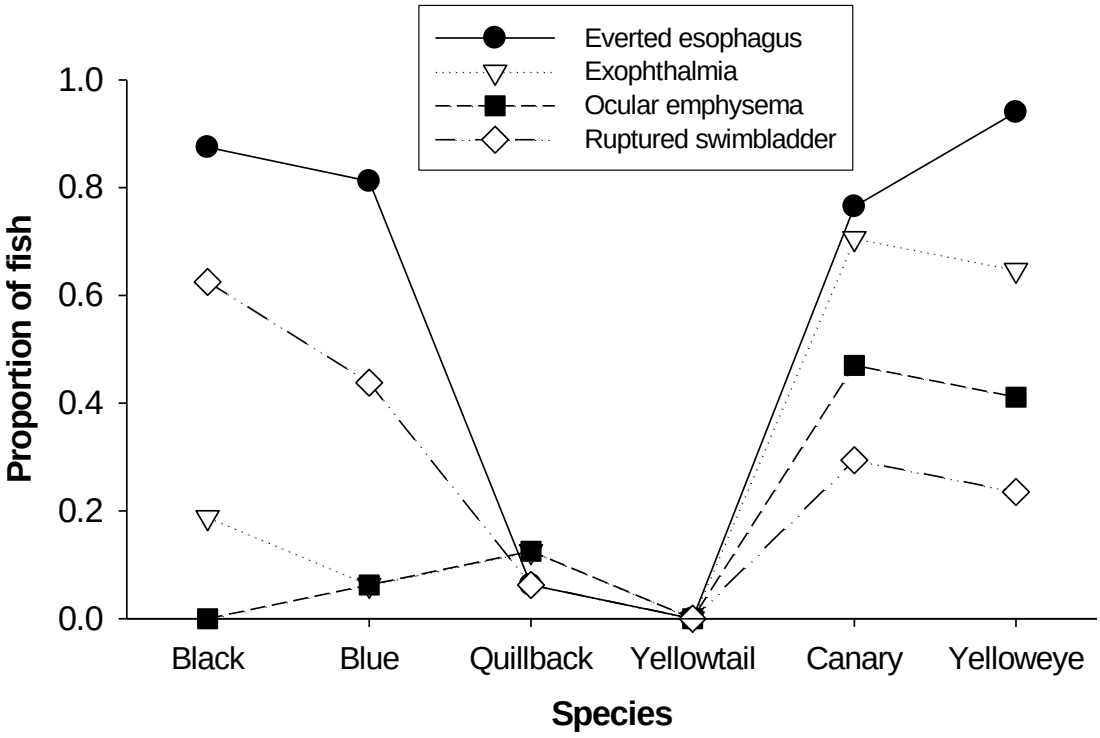


Figure 1. Proportion of rockfish with selected macroscopic barotrauma indicators. Connecting lines do not represent data and are for visual purpose only.

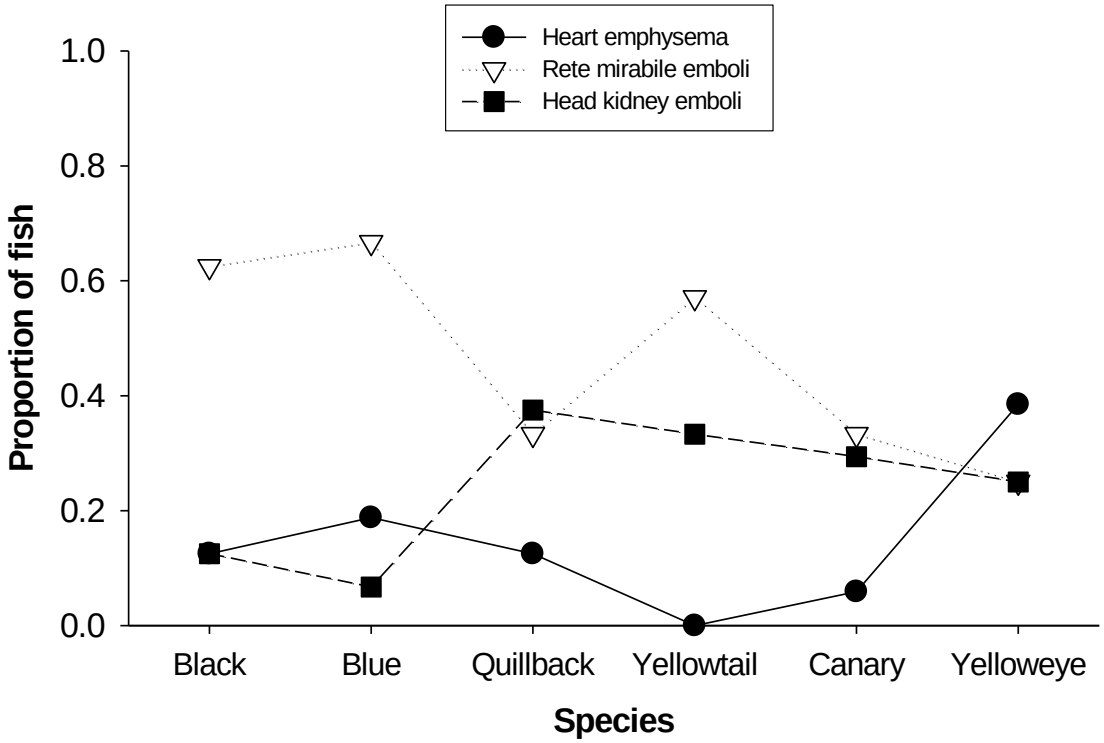
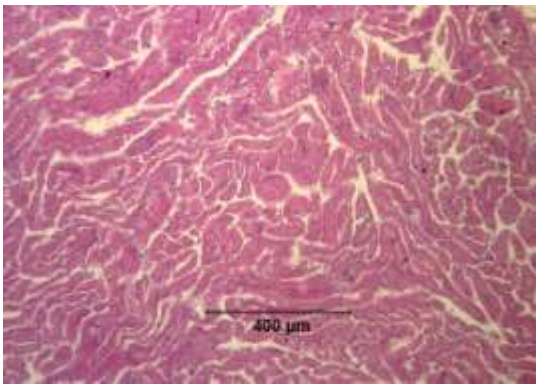


Figure 2. Proportion of rockfish with histologic barotrauma indicators. Connecting lines do not represent data and are for visual purpose only.

Figure 3. Histology of barotrauma injuries in the heart, rete mirabile and head kidney of rockfish. A) Normal heart ventricle. B) Tissue emphysema in the heart ventricle. C) Normal rete mirabile. D) Emboli in the rete mirabile. E) Normal head kidney vessel. F) Emboli in a vessel of the head kidney.

A.



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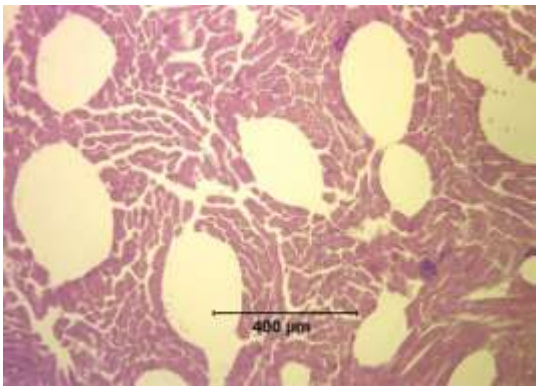

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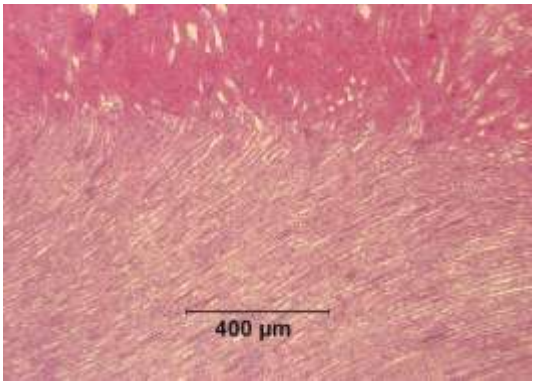

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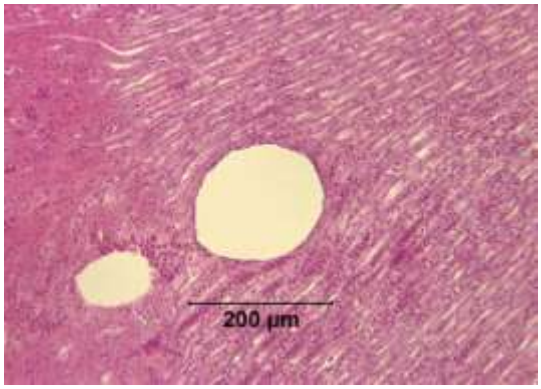

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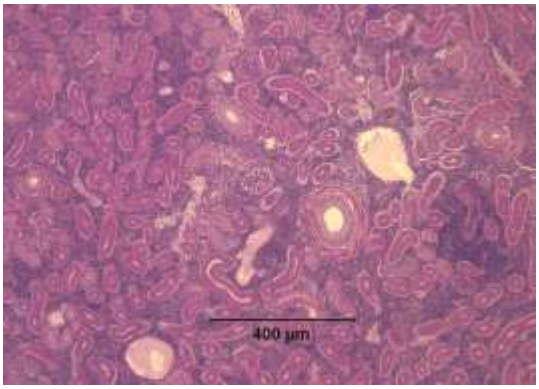

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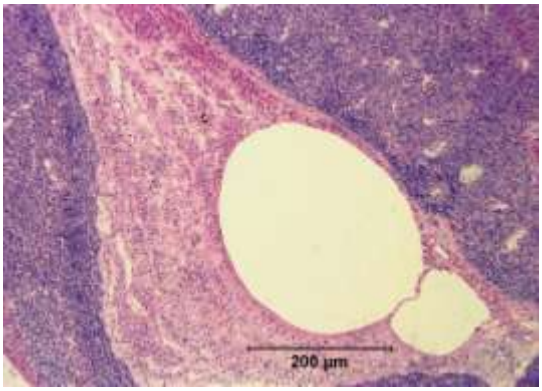
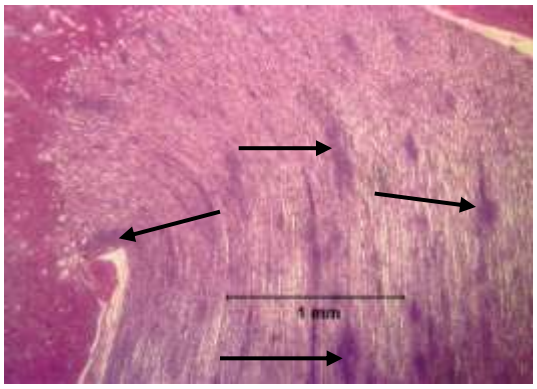


Figure 3.

Figure 4. Histology of inflammation and parasite infections in the heart, rete mirabile, head kidney, and liver of rockfish. A) Inflammation in the rete mirabile. B) Heart ventricle infected with Digenean trematodes. Arrows indicate trematodes. C). Active and degraded Ichthyophonous in the head kidney. D) Mycobacterium-like granuloma in the head kidney. E) Myxozoan spores in the liver. F) Larval nematodes in the liver. Arrows indicates areas of inflammation and/or infection

A.



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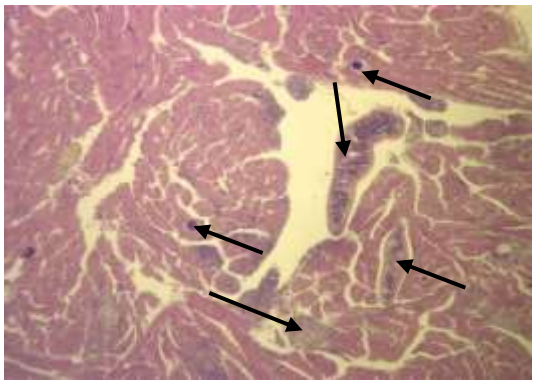

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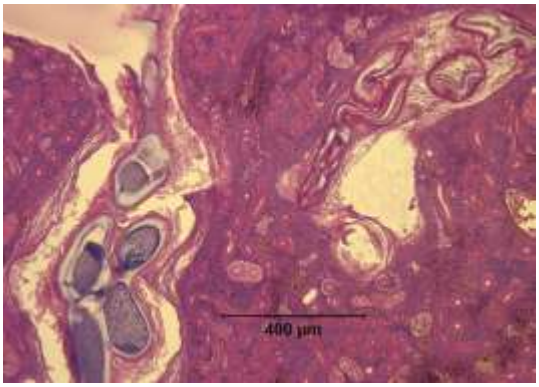

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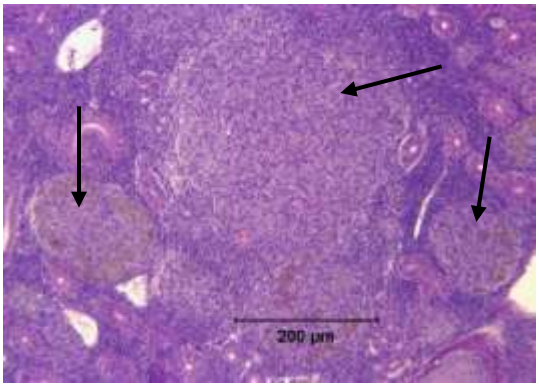

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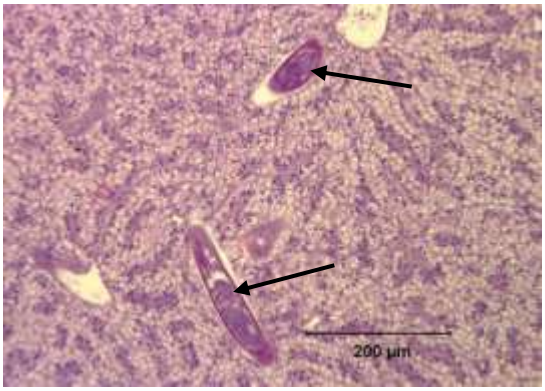
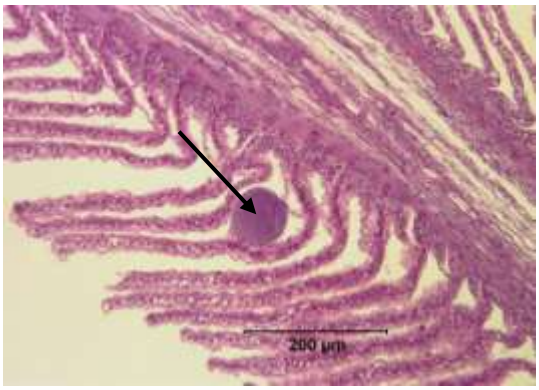


Figure 4.

A.



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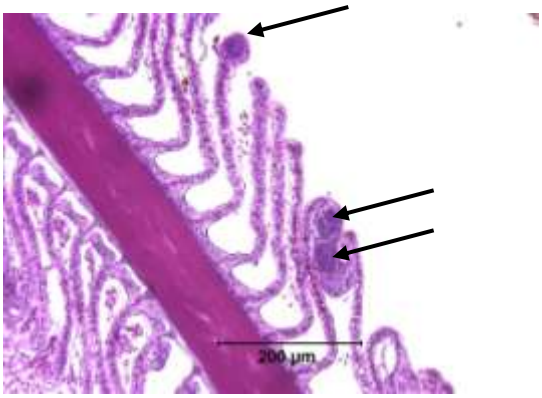

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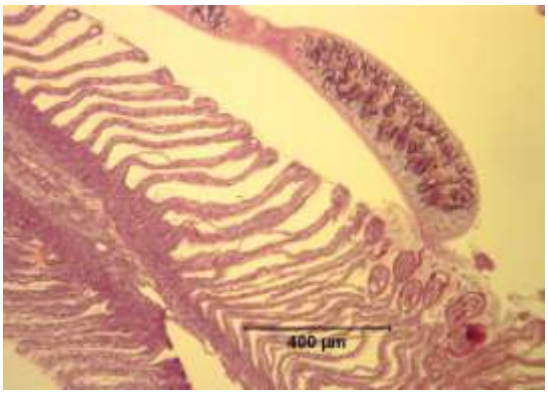

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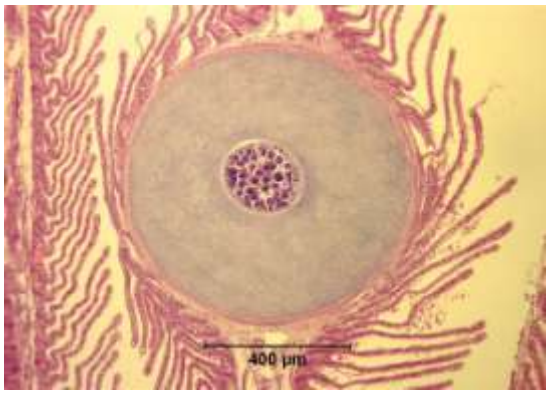

Figure 5. Histology of parasite infections in the gills of rockfish. A) Epitheliocystis. B) Blood fluke eggs. C) Monogenean parasite. D) Cyst of unknown etiology. Arrows indicate areas of infection.

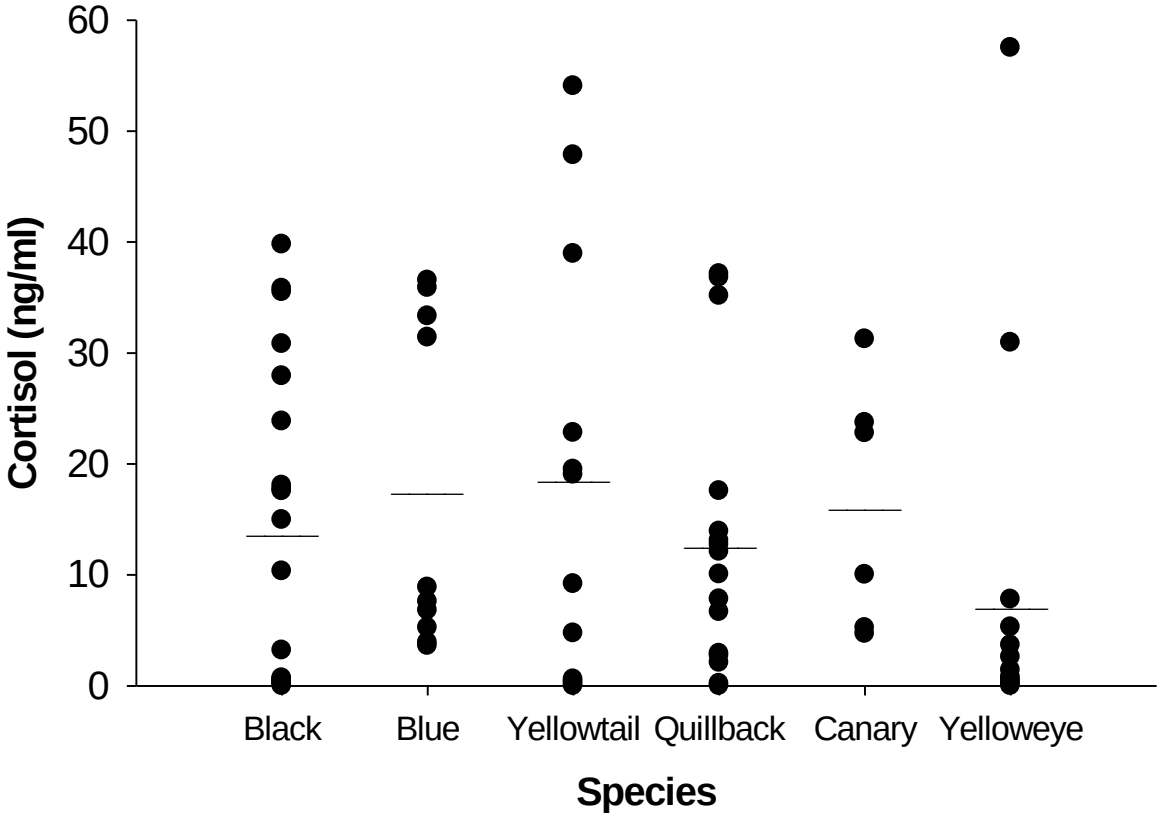

Figure 6. Individual cortisol values for each species of rockfish. Means are represented by the horizontal bar.

Recovery potential of black rockfish (*Sebastes melanops*) following forced decompression and subsequent recompression

Alena L. Pribyl, Carl B. Schreck, Kevin E. Kelley, Michael L. Kent, and Steven J. Parker

Introduction

Pacific rockfish (*Sebastes* spp.) are physoclists, which means they have a closed swimbladder from which gas cannot escape unless it is torn or ruptured. This functions well for fish species inhabiting deep water because they can inflate their swimbladder at whichever depth they choose and are not limited to gulping air from the surface. However, when rockfish are captured and undergo a forced ascent, gas in the closed swimbladder expands as pressure is decreased (Boyle's Law), causing a variety of pressure-related injuries collectively called barotrauma. Depending on the degree of pressure change, barotrauma in physoclist fish can involve bloating, ruptured swimbladder, crushed organs, eversion of the esophagus or stomach, and exopthalmia (Gotshall 1964, Hannah et al. 2008a, Jarvis and Lowe 2008, Parker et al. 2006, Pribyl et al. 2009, Rummer and Bennett 2005).

Rockfish are captured in both recreational and commercial fisheries, and bycatch of non-target rockfish is a common problem (Parker et al. 2000). Rockfish occur in mixed-species assemblages and often have similar feeding habits, thus targeting a given species is difficult. In multi-species fisheries, small stocks or less resilient stocks are vulnerable to overexploitation. Currently there are seven species of rockfishes classified as "depleted" by the Pacific Fishery Management Council (PFMC 2008). Recovery of these populations is expected to be slow because of long generation times and sporadic recruitment (Parker et al. 2000).

Management tools for rockfish are currently limited to restricting fishing effort, and/or requiring discard of the incidentally caught species of concern (Parker et al. 2000). Discarding these species is required so they will not be targeted. A concern with this management strategy is that many rockfishes are believed to have high mortality rates when discarded because of barotrauma injuries incurred during capture and/or from the inability to return to depth due to excessive buoyancy and exhaustion (Parker et al. 2000).

Recompression is a technique that has been used by some recreational fishermen to return discarded rockfish back to depth. Recompression involves any method that will help the rockfish overcome its buoyancy to submerge to a depth close to its original capture. If a rockfish can submerge close to its original capture depth, the expanded gases will compress again, relieving the fish of its excessive buoyancy. Devices that are often used to recompress rockfish include barbless, weighted hooks and weighted cages (Theberge and Parker 2005). Recent studies have shown these devices are effective at increasing the short-term survival of rockfish with barotrauma (Hannah and Matteson 2007, Jarvis and Lowe 2008), however little is known about the long-term survival of recompressed rockfish. Thus far, only one study has investigated long-term survival in black rockfish, however the focus was on mortality and the healing rate of swimbladders (Parker et al. 2006). No study has undertaken a complete macroscopic and physiologic evaluation of recovery in rockfish after recompression.

We investigated the 31-day survival and recovery of black rockfish (*S. melanops*) that underwent simulated decompression from 35 m followed by recompression using hyperbaric pressure chambers. The objective of this study was to determine the potential for long-term recovery in black rockfish decompressed and recompressed from 35 m by using both macroscopic and physiologic measures. Although black rockfish are not considered depleted, they are long-lived and are commonly captured in the recreational fishery. Black rockfish also commonly experience a wide array of both macroscopic and tissue-level barotrauma indicators. Black rockfish decompressed from depths near 35 m often experience everted esophagus, exophthalmia, and ruptured swimbladder (Hannah et al. 2008a, Parker et al. 2006, Pribyl et al. 2009), and at the tissue level black rockfish can experience emphysema in the heart ventricle, emboli in the rete mirabile and emboli in the vessels of the head kidney (Pribyl et al. 2010, *in preparation-a,* Pribyl et al. 2009). By observing the recovery process at both the macroscopic and physiologic level over a month long recovery period, we can gain a better understanding of a rockfish's potential for long term recovery.

Methods

Fish collection

We collected 60 adult black rockfish off the coast of Newport, Oregon by hook and line from depths less than 15.2 m. Each rockfish was individually tagged using PIT tags as

described in Parker and Rankin (2003). Only rockfish with no or minimal indicators of barotrauma (air in pharyngo-cleithral membrane) were utilized. Total lengths ranged from 36 to 49 cm, which is generally the length at first maturity (Love et al. 2002), thus we classified these fish as adults. Upon return to Newport, rockfish were immediately transferred into 2.4 m diameter flow-through tanks (106,000 L) at the Hatfield Marine Science Center (HMSC) where fish were held until neutrally buoyant and actively feeding (minimum 30 days). Cessation of feeding is a common response to stress (physical or perceived) and resumption of feeding can be an indicator that fish have recovered from the stress (Rice 1990). Neutral buoyancy indicates the swimbladder is functioning. Fish were held for a minimum of 30 days as a precaution to ensure recovery from any minor stressors as described in Parker et al. (2006). Other studies (McElderry 1979, Parker et al. 2006) have used this collection and holding technique on hundreds of black rockfish and no noticeable effect from capture was seen in control fish in these studies.

Fish density was 10 - 15 fish per tank and flow rate was 12 - 15 L/min. Dissolved oxygen (> 80% saturation), salinity (range: 34 – 37 ppt), and temperature (range: 9.4 - 14.4 °C) were monitored daily. Rockfish were fed a diet of thawed Atlantic silversides *Menidia menidia*, pink shrimp *Pandalus jordani* and California market squid *Loligo opalescens* three times a week and tanks were cleaned by siphoning debris daily.

Recompression Experiments

For each recompression experiment, six black rockfish were placed in each of two large flow-through hyperbaric pressure chambers (8,700 L) and adjusted to 4.5 ATA (approximately 35 m depth) following a standard protocol as described in Parker et al. (2006). The pressure chambers are described in detail in Parker et al. (2006). The 35 m simulated depth is within the depth range black rockfish are commonly captured in the recreational fishery. Fish were neutrally buoyant within 10 days. One chamber served as a treatment chamber and the other chamber served as a control chamber. Once neutrally buoyant, fish in the treatment chamber were exposed to a simulated capture event by decreasing pressure to 1 ATA (0 m) over a 90 sec period to induce decompression. After the simulated capture, the fish were held at surface pressure for 3 min to simulate the time it takes to unhook the fish and place it in a recompression device. The fish were then immediately recompressed (30 sec) to 4.5 ATA. Control fish remained at 4.5 ATA during this time. After a minimum recovery time of 6 hours post-recompression for treatment fish, both treatment and control fish were slowly brought to surface pressure with a 10% pressure reduction every 2 – 3 hours over a period of 72 h. This rate of ascent was determined by McElderry (1975) as slow enough for black rockfish to adjust to with no physical damage. Observations of neutral buoyancy at each pressure change confirmed that fish could adjust physiologically to this rate of ascent.

Sample collection

Once at surface pressure, two rockfish from each chamber were immediately removed from the pressure chambers, examined for external barotrauma indicators, and sampled for blood via a caudal venipuncture using heparinized BD Vacutainers® (Becton, Dickinson and Company, Franklin Lakes, NJ). External barotrauma indicators were recorded followed Hannah et al. (2008) and included: everted esophagus, swollen abdomen, inflated pharyngo-cleithral membrane, air bubbles in the pharyngo-cleithral membrane, exophthalmia, and ocular emphysema. Blood samples were immediately placed on ice and centrifuged (1000 G x 6 min) within 15 minutes of collection; plasma was then stored at -80 ºC. Following blood sampling, rockfish were euthanized in an overdose of tricaine methane sulfonate (MS-222) and then the following organs were collected: eye, liver, head kidney, gonad, rete mirabile and heart ventricle. All fish were dissected within 20 minutes of euthanization. Tissue samples were fixed in Davidson's solution (Kent and Poppe 1998) at a ratio of no more than 1:10 (tissue:solution). During dissection, fish were also examined for ruptures in the swimbladder and hemorrhaging.

The remaining four treatment rockfish and four control rockfish were transported to the 2.4 m diameter tanks described above for recovery. Two treatment and two control fish each, were subsequently sampled as described above at 15 and 31 days post-decompression. We used a 31-d experimental period because it allowed us to evaluate longer-term survival and still complete several replicates of the experiment.

This experiment was replicated five times, for a total of 30 treatment fish and 30 control fish, or a total of 10 fish sampled per treatment and sample day (Figure 1).

In addition, plasma from ten black rockfish sampled from a boat using hook and line were used to establish non-stressed plasma values of black rockfish. These rockfish were captured from depths ranging between 12 m to 18 m and were immediately sampled for blood with a caudal venipuncture using heparinized BD Vacutainers®. Blood samples were collected in less than 5 min from initial hooking, thus although the fish experienced capture stress and some barotrauma, concentrations of many enzymes and proteins in the blood will not have had time to respond to the effects of capture. Because these fish were sampled within 5 minutes of being removed from natural conditions, we believe these samples come as close as possible to reflecting baseline conditions in the wild. We refer these samples as our "field baseline" throughout the paper. These blood samples were processed as described above.

Sample Processing

Plasma samples were thawed and analyzed for plasma enzymes indicative of tissue injury, general metabolites, insulin-like growth factor-1 (IGF-1), and cortisol. Plasma enzymes measured included: lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and alkaline phosphatase (ALP). Metabolites measured included: glucose, sodium, chloride, potassium, calcium,

and phosphorous. Both plasma enzyme and metabolite measurements were conducted at Oregon State University's Veterinary Diagnostic Laboratory on a Hitachi 917 chemical analyzer. Cortisol was analyzed using a radioimmunoassay following procedures in Redding et al. (1984). Insulin-like growth factor-1 was analyzed using a radioimmunoassay according to Moriyama et al. (1994).

Only plasma from the first three replicates was analyzed for plasma enzymes because of the high cost of the assays and because it became evident that high variability in both experimental and field baseline samples would preclude any detection of significant differences between our field baseline and experimental groups. For metabolites, only plasma from day 3 and day 31 rockfish was analyzed because we were primarily interested in the overall change over time. All plasma samples were analyzed for IGF-1 and all samples except for the first replicate (insufficient plasma was collected during the first replicate) were analyzed for cortisol.

Tissues were fixed for a minimum of 30 d, embedded in paraffin, and then sectioned to a thickness of 5-7 μm. For the eye, slides were sectioned by Oregon State University's Veterinary Diagnostic lab, with a vertical cross section that showed the entire circumference of the eye including the cornea, iris, lens, retina, choroid and sclera. Slides were stained with heamatoxylin and eosin Y. Slides were evaluated with a Leica compound light microscope (Model DM LB; Leica Microsystems, Wetzlar, Germany).

Statistics

Measurements from the two pseudoreplicate fish within each experimental replicate were averaged because of tank effects for three of the metabolites. This pooling of pseudoreplicates yielded a final sample of n = 5 per treatment and sample day, although the sample number represents twice as many fish. For blood enzyme data, because only samples from the first three replicates were measured, this yielded n = 3 per treatment and sample day. For plasma cortisol, this yielded n = 4 per treatment and sample day. A Welch's test was used to test for differences between treatment, control, and field baseline groups because of unequal sample variances between groups. Dunnett's post-hoc test was used to determine between which groups there were differences.

To determine if there was a difference in blood plasma measurements as a result of the presence or absence of variables such as ruptured swimbladder, ruptured tunica externa, feeding, and inflammation in the rete mirabile, we used Mann-Whitney tests and logistic regression models. We found similar results using both methods, thus for simplicity, we only present results from the Mann-Whitney tests here. For these calculations we used measurements at the level of the individual fish because we were not determining differences between fixed treatment groups (n = 60 for IGF-1, n = 48 for cortisol, n = 40 for metabolites, and n = 36 for enzymes). Because of the multiple tests we ran utilizing data from the same experimental unit (ie: individual fish) we also applied a correction to

control for the false discovery rate as described in Waite and Campbell (2009). We used nonparametric tests because of non-normal distributions in several blood plasma analytes. All analyses were conducted on SPSS v.17.0.

Results

Macroscopic observations

During decompression in the hyperbaric pressure chambers, we observed treatment rockfish exhibited the macroscopic barotrauma indicators of everted esophagus and exophthalmia. While we estimate about 80% of the fish experienced an everted esophagus and about 50% experienced exophthalmia, we could not get an accurate count of the number of fish with different barotrauma indicators because the fish were moving rapidly during the surface interval. Once treatment fish were recompressed to their original depth, all external indicators of barotrauma disappeared. The only indication that treatment fish had experienced barotrauma was that most fish were negatively buoyant. We did not observe any indication of barotrauma or negative buoyancy in control rockfish during or after the experiments.

No mortalities occurred in treatment or control fish for the duration of the experiments (n = 60). By day 31, 80% of both treatment and control fish had resumed feeding. There was also no difference in the resumption of feeding between treatment and control fish prior to day 31. No external injuries were visible on treatment or control fish over the

entire sampling period. Once dissected, the only macroscopic injury we observed was the presence of a ruptured swimbladder and/or a ruptured tunica externa (the outer layer of the swimbladder) in treatment fish. The proportion of treatment fish with a ruptured swimbladder was 80% at day 3 post-decompression, and declined to 20% and 50% at days 15 and 31 post-decompression. The proportion of treatment fish with a ruptured tunica externa was 100% at day 3 post-decompression, and only declined to 80% at days 15 and 31 post-decompression. No control fish had a ruptured swimbladder or a ruptured tunica externa.

Histology

The rete mirabile was the only organ to show injury at the histological level (Figure 2). Severe injury (emboli, hemorrhaging) of the rete mirabile was present in two of 30 treatment fish; one of the fish was sampled 3 days post-decompression and the other fish was sampled 15 days post-decompression. Chronic, multifocal inflammation of the rete mirabile was present in both treatment and control fish throughout the recovery period. Eye, head kidney, liver, heart ventricle, and gonad showed no injury due to forced decompression.

Blood Plasma Analyses

Blood plasma analyses of enzymes indicative of tissue injury showed high variability in treatment, control and field baseline samples (Table 1). There were no differences

between treatment, control, or field baseline groups for LDH, AST, ALT, and ALP within each sample day (Welch's test: $P > 0.14$ for all 12 tests; df = 2). There were also no differences between treatment, control and field baseline groups for CK at days 15 and 31, however at day 3 the control group was elevated relative to the field baseline group (Dunnett's test, $P = 0.022$). There were no differences between the treatment and control group, or the treatment and field baseline group (Dunnett's test, $P > 0.7$ for both tests).

The metabolites glucose, sodium, chloride, potassium, calcium, and phosphorous showed no difference between treatment and control groups within sample day 3 and sample day 31 (Dunnett's test, $P > 0.5$ for all 12 tests). There were differences between treatment and/or control groups and the field baseline group though (Table 2). Glucose levels for the day 3 control group and day 31 treatment and control groups were elevated compared to the field baseline group (Dunnett's test, $P < 0.05$ for all three tests). Potassium levels for the day 31 treatment group was depressed relative to the field baseline group (Dunnett's test, $P = 0.001$). Calcium levels at day 3 for both treatment and control groups were depressed relative to the field baseline group (Dunnett's test, $P < 0.05$ for both tests), however showed no differences by day 31 (Dunnett's test, $P > 0.4$ for both tests).

Levels of IGF-1 (Figure 3) showed no differences between treatment and control groups within each sample day (Dunnett's test, $P > 0.2$ for all three tests), however for day 3, the treatment group was different than the field baseline group (Welch's test, df =2, $P = 0.048$; Dunnett's test, $P = 0.056$). Levels of cortisol (Figure 4) also showed no differences between treatment and control groups within each sample day (Dunnett's test: $P > 0.1$ for all three tests), however cortisol levels in the control group were elevated relative to the field baseline group on days 15 and 31 (Dunnett's test, $P < 0.05$ for both tests).

Analyses to determine if the presence or absence of injury (ruptured swimbladder, ruptured tunica externa, inflammation in the rete mirabile, or feeding) affected levels of blood plasma analytes yielded interesting results (Table 3). Fish with inflammation in the rete mirabile had lower cortisol levels compared to fish with no rete mirabile inflammation. We also found fish that were feeding had higher levels of CK, glucose, calcium, phosphorous and cortisol compared to fish that were not feeding (Table 3).

Discussion

Results from controlled decompression/recompression experiments show that black rockfish decompressed from 4.5 ATA have the potential to survive 31-days post barotrauma if recompressed, however they will face reductions in fitness compared to un-captured fish. Indicators at the macroscopic to microscopic level such as resumption in

feeding and no differences in blood plasma analyte levels between treatment and control fish, suggest there is the potential for longer-term recovery in black rockfish; however injuries to the swimbladder and rete mirabile that are slow in healing will cause a reduction in fitness and may affect longer term survival in the wild.

At the macroscopic level, cessation of feeding in fish is a common response to physical or perceived stress (Rice 1990) and resumption of feeding can be an indicator that fish have at least partially recovered from the stress. Because we observed no differences in feeding behavior between treatment and control fish, this seems to indicate the additional stress of barotrauma does not contribute significantly to the recovery of feeding behavior.

The primary injury at the macroscopic level was the presence of a ruptured swimbladder and/or tunica externa. By days 15 and 31 post-decompression, only 50 to 80% of swimbladders had healed enough to hold gas and the majority of fish still had a ruptured tunica externa. Parker et al. (2006) observed similar healing rates when they decompressed and recompressed black rockfish from 4.0 ATA using pressure chambers. They observed 100% of rockfish had ruptured swimbladders immediately after the decompression/recompression regime and observed 77% of the swimbladders had healed enough to hold gas after a 21 day recovery period. This healing rate at 21 days post-decompression is similar to our observations and our combined data suggests there will be a percentage of rockfish that will take an extended period of time to heal their

swimbladder, or may not be able to heal their swimbladder at all. We also observed that although many swimbladders are healing enough to hold gas over time, the outer layer of the swimbladder, the tunica externa, did not heal in 80% of fish after one month, suggesting the tunica externa will take much longer to heal. Rockfish with swimbladders that are unable to hold gas will not be able to maintain neutral buoyancy in the water column and will likely need to expend more energy to find prey and escape predation. In addition, a ruptured swimbladder could affect the ability of rockfish to communicate. Rockfish use their swimbladder for sound production, often in reponse to agnostic encounters and for territorial defense (Hallacher 1974, Sirovic and Demer 2009). In other fish species, sounds are also produced during feeding and mating (Myrberg and Lugli 2006). Thus, a ruptured swimbladder could affect a rockfish's ability to interact socially. Rockfish with a ruptured tunica externa may also have difficulties with sound production because gas may be able to diffuse through the swimbladder walls more easily, thus inhibiting a rockfish's ability to keep the swimbladder inflated at the desired level. Because rockfish in our experiment were able to recover in predator-free and prey-plentiful conditons, the degree to which these injuries reflect survival in the wild is unknown. Survival of compromised rockfish will likely be dependent on conditions in the ocean at the time of release.

At the tissue level, we found no injury in the heart ventricle, eye, head kidney, liver, or gonad. These organs have been affected by decompression and/or gas bubble disease in

other fish species (Beyer et al. 1976, D'Aoust and Smith 1974, Feathers and Knable 1983, Longbottom 2000, Pribyl et al. 2009). Black rockfish decompressed from 35 m and greater have experienced emphysema in the heart ventricle after decompression (Pribyl et al. 2010, *in preparation-a,* Pribyl et al. 2009). In Pribyl et al. (2009), over half of black rockfish that were decompressed from 4.5 ATA had emphysema, or gas bubbles, in the heart ventricle. We did not observe any evidence of this in rockfish sampled 3 days post-decompression or later. One possibility is that emphysema in the heart ventricle is a minor injury and was able to heal within the three-day period post-decompression. It is known that zebrafish (*Danio rerio*) can regenerate injured cardiac muscle (Lepilina et al. 2006, Poss et al. 2002, Raya et al. 2003) and the same may be true for rockfish. Lepilina et al (2006) showed that three days after amputating the apex of the heart ventricle in zebrafish, myocardial progenitor cells had already flooded the amputation site. Emphysema in the heart ventricle is on a much smaller scale than the amputation described in Lepilina et al. (2006), and we speculate it is possible for rockfish myocardium to heal itself from minor emphysema within the three day time period.

No previous study has examined the rockfish eye histologically after barotrauma. We found it remarkable that even though we observed many fish with exophthalmia, we did not observe any injury at the histological level. We expected to find evidence of embolism or tissue displacement as a result of the exophthalmia. However, histology only evaluates a small amount of the total volume of the eye and a section may have

simply missed damaged tissue. Research on eye function in black rockfish with exophthalmia however, does support our findings (Brill et al. 2008). Brill et al. (2008) used electroretinography to measure changes in retinal light sensitivity, flicker fusion frequency, and spectral sensitivity in black rockfish with exophthalmia that were decompressed and recompressed from 3.0 ATA in pressure chambers. He found no indication that rapid decompression and associated exophthalmia had any effect on rockfish retinal function. Brill et al. (2008) did note though, that their procedures would not have detected any damage to the optic nerve or other parts of the nervous system associated with vision. The histological cross sections of the eye analyzed in our study did not include the optic nerve either, thus we were also unable to detect if there was any injury to the optic nerve. Rogers et al. (2008) did examine the optic nerve in rockfish with exophthalmia using magnetic resonance imaging (MRI) and found severe exophthalmia caused extreme stretching of the optic nerves and gas build-up in an orbital space behind the eye. This means that although we found no injury to the eye itself, there could still be vision problems in rockfish associated with stretching of the optic nerve.

We also found no evidence of injury due to barotrauma in the head kidney, liver, or gonad. Previous work by Pribyl et al. (2009) also showed no evidence of injury in these organs following decompression from 35 m in black rockfish, although rockfish decompressed from greater depths (Pribyl et al. 2010, *in preparation-a*) exhibited emboli

in the vessels of the head kidney. No other work has evaluated rockfish gonads histologically after decompression.

The rete mirabile was affected by decompression in a small percentage of rockfish. This organ is a gas-concentrating organ that resides in the secretory portion of the rockfish swimbladder and consists of a large network of venous and arterial capillaries. Gas from the venous capillaries diffuses into the arterial capillaries, increasing the gas partial pressure. Blood then flows from the rete capillaries into the gas gland where secretions of lactic acid and carbonic anhydrase increase the dissolved gas partial pressures even more by way of the Root effect (Evans 1998). The high partial pressure of dissolved gas then diffuses into the secretory portion of the swimbladder and inflates it. For the two rockfish we observed with severe damage to the rete mirabile, it is unlikely the rete mirabile is functional because most of the capillaries were destroyed by hemorrhaging and emboli. It is thus unlikely that these fish would be able to inflate their swimbladder (if it healed) and achieve neutral buoyancy. In earlier work (Pribyl et al. 2010, *in preparation-a*) we found a small proportion of black rockfish sampled immediately after decompression from depths greater than 35 m also had emboli (although there was no hemorrhaging) in the rete mirabile. In this study we only observed emboli in the the two rockfish mentioned above. It is possible a greater number of rockfish had emboli in the rete mirabile in this study, but the emboli healed within the three-day recovery period prior to sampling. Chronic, multifocal inflammation in the rete mirabile was also present

in both treatment and control fish and appears to be an underlying condition in many rockfish not associated with barotrauma (Pribyl et al. 2010, *in preparation-a*). The cause of rete mirabile inflammation is unknown. Rockfish are infected with many parasites and bacteria, all of which could cause chronic inflammation, however in Pribyl et al (2010, *in prepararation-b*), special stains did not reveal any parasites or bacteria present in the rete mirabile near areas of inflammation. Additionally, Pribyl et al. (2010, *in prepararation-b*) observed that inflammation primarily occurred in black rockfish and yellowtail rockfish, two species known to undergo extensive vertical migrations (Parker et al. 2008, Pearcy 1992), which suggests this condition may be related to frequent changes in depth. It is unclear if this inflammation will significantly affect a rockfish's ability to inflate its swimbladder or affect its overall fitness.

Plasma enzymes indicative of tissue damage have been measured in several fish species after physical trauma. These enzymes are released when there is injury to certain tissues, and many of these enzymes are concentrated in the heart and liver. Morrissey et al. (2005) found a significant elevation in levels of AST, LDH, and creatine phosphokinase (CPK) in smallmouth bass *Micropterus dolomieu* with barotrauma compared to bass without barotrauma. Wagner and Congleton (2004) found that ALT, AST, CK, and LDH measured in juvenile chinook salmon from a fish bypass or from a transport barge grouped together in a factor analysis for tissue damage. These studies seem to indicate that the enzymes ALT, AST, CK and LDH should respond to a physical stressor in fish.

However, we did not see any significant differences in LDH, ALT, CK, ALP or AST between treatment and control groups, and only in one case, was there a difference between treatment or control groups and the field baseline group. We also did not observe a difference between enzyme activity and presence or absence of tissue injuries such as ruptured swimbladder and rete mirabile inflammation. One possible explanation is these enzymes do not reside in high concentrations in swimbladder tissue, and the tissues where the enzymes are highly concentrated (mostly the muscle, heart and liver) were uninjured in our experiments. Another possibility is that our sample size was too low to detect any differences due to high variability among samples. It is interesting to note though, that even our field baseline samples from wild-caught rockfish showed high variability. Thus, plasma enzymes may not be a good plasma indicator for stressed black rockfish when sample sizes are low.

We observed no differences in metabolite concentration between treatment and control fish, indicating that differences between experimental fish and field baseline values were likely due to handling and feeding stress incurred during the experimental procedures instead of barotrauma. We were unable to feed rockfish while they were in the pressure chambers, thus both treatment and control rockfish experienced a two-week fasting period. Plasma glucose is part of the primary and secondary stress response and levels can increase quickly following a stressor. The handling of fish prior to sampling was likely the cause of elevated glucose levels observed in both treatment and control fish

relative to field baseline levels. Fish sampled on day 31 likely had higher glucose levels than fish sampled earlier because they had resumed feeding; Olsen et al. (2008) found that plasma glucose levels were higher in Atlantic cod *Gadus morhua* that had been fed and subjected to an acute stressor compared to cod that had been food deprived and subjected to an acute stressor. Calcium levels were depleted in experimental fish at day 3 but returned to field baseline values by day 31. This is also likely due to the fasting period all experimental fish experienced; about half of plasma calcium is bound to plasma proteins (Andreasen 1985, Björnsson et al. 1989), thus a decline in calcium would be expected after fasting because protein levels would be depressed. Indeed, we found differences in glucose, calcium and phosphorous levels between fish that were feeding and fish that were not feeding.

The hormone IGF-1 has been correlated with growth rate in several fish species including barramundi *Lates calcarifer*, Atlantic salmon *Salmo salar*, Coho salmon *Oncorhynchus kisutch*, Arctic charr *Salvelinus alpinus*, and Southern Bluefin tuna *Thunnus maccoyii* (Beckman et al. 2004, Cameron et al. 2007, Dyer et al. 2004). Thus, IGF-1 has the potential to be a good indicator of normal growth for teleost fish. IGF-1 levels in rockfish from this study did not differ between treatment, control, and field baseline values, indicating growth was not perturbed by the decompression/recompression treatment.

Cortisol is the primary corticosteroid produced in teleost fish and is part of the primary stress repsonse (Wendelaar Bonga 1997). Because cortisol levels tend to increase dramatically from baseline levels during a stress response, cortisol has been widely used as a stress indicator for fish. We observed elevated cortisol levels in both treatment and control fish relative to field baseline levels at each sample day, indicating experimental fish were stressed throughout the recovery period, although the variance was high. We also found fish with no rete mirabile inflammation had higher levels of cortisol than fish with inflammation. This could be a result of the anti-inflammatory properties of cortisol (Castillo et al. 2009, MacKenzie et al. 2006) where rockfish with higher cortisol levels may experience reduced inflammation. We also observed cortisol levels were higher in fish that were feeding; this is unusual as elevated cortisol levels are typically associated with fish that are not feeding (Bernier 2006, Olsen et al. 2008, Pankhurst et al. 2008). We are uncertain why our fish show this difference, except that perhaps the cortisol levels are reflective of the capture stress prior to sampling. It is possible fish that were feeding had more energy and were more difficult to capture than fish that were not feeding, thus the increased time it took to capture fish that were feeding could have allowed cortisol levels to increase.

This study shows that black rockfish subjected to decompression from 35 m with subsequent recompression have the potential for long-term survival, however the degree of recovery and number of fish surviving will likely be dependent upon ocean conditions.

Blood plasma analyses seem to indicate no strong effects due to barotrauma, although organ histology indicates a minority of rockfish may experience severely damaged rete mirabiles, and gross observation indicates 20 – 50% of rockfish will not be able to heal their swimbladders after one month. Rockfish with ruptured swimbladders and/or an injured rete mirabile may face a compromised ability to find prey and escape predation, and they may not be able to effectively communicate with other rockfish, although the extent to which this will affect survival is unknown. It is important to note that our rockfish were able to recover in a predator-free zone with plenty of food available. In the wild, rockfish may face more difficulties, especially rockfish with compromised buoyancy. Long-term tagging studies would be necessary to estimate the degree to which injuries such as a ruptured swimbaldder would affect survival. Altogether, this study shows an incredible resiliency in the ability of rockfish to survive barotrauma. Although recompressed rockfish may not recover completely, this study indicates that recompressing rockfish captured up to 35 m of depth is still likely to provide rockfish with a chance at survival they might not otherwise have.

Acknowledgements

We thank Polly Rankin from the Oregon Department of Fish and Wildlife (ODFW) who assisted and taught us fish collection, fish husbandry, and operation of the hyperbaric pressure chambers. We thank the numerous personnel at ODFW (Newport, OR) who assisted in fish collections and sample collections. We also thank Cliff Pereira and John

Vansickle for assistance with statistics. This research was funded by the Coastside Fishing Club (San Francisco, CA), ODFW (Newport, OR), the Mamie Markham Fund (HMSC), the Department of Fisheries and Wildlife at Oregon State University (OSU), and the NOAA Saltonstall-Kennedy Grant.

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Table 1. Mean value ± SE for blood plasma enzymes in black rockfish, according to sample day and treatment (n = 3 for each day/treatment). Field baseline means from field-captured rockfish are in bold (n = 10). LDH = lactate dehydrogenase, ALP = alkaline phosphatase, CK = creatine kinase, AST = aspartate aminotransferase, ALT = alanine aminotransferase.

Sample day, treatment	LDH (IU/L)	ALP (IU/L)	CK (IU/L)	AST (IU/L)	ALT (IU/L)
Field Baseline	**75.4 ± 19.2**	**18.3 ± 2.0**	**83.3 ± 15.2**	**18.5 ± 4.1**	**3.7 ± 1.7**
03, control	91.6 ± 32.6	17.3 ± 2.0	354.8 ± 339.1	16.2 ± 6.9	4.5 ± 4.5
03, treatment	23.9 ± 13.8	17.2 ± 3.0	10.5 ± 2.4	6.5 ± 3.9	0.7± 0.7
15, control	111.6 ± 62.9	26.2 ± 6.6	96.8 ± 82.5	17.7 ± 8.7	1.3 ± 1.3
15, treatment	42.7 ± 15.5	19.2 ± 2.9	153.7 ± 148.9	6.7 ± 3.9	1.7 ± 1.7
31, control	58.7 ± 33.8	28.8 ± 5.0	539.3 ± 307.6	17.2 ± 9.1	4.7 ± 2.7
31, treatment	67.7 ± 23.9	22.2 ± 1.5	616.2 ± 324.3	17.0 ± 4.5	8.3 ± 3.8

Table 2. Mean value ± SE for blood plasma metabolites in black rockfish, according to sample day and treatment (n = 5 for each row). Field baseline means from field-captured rockfish are in bold (n = 10). Na = sodium, K = potassium, Cl = chloride. * = difference at the 0.05 level between the specified value and the field baseline value (Dunnett's test).
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Sample day, treatment	Glucose (mg/dl)	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Calcium (mg/dl)	Phosphorous (mg/dl)
Field baseline	**28.9 ± 1.6***	**187.6 ± 1.1**	**3.5 ± 0.1***	**155.6 ± 1.1**	**12.3 ± 0.3***	**8.5 ± 0.5**
03, control	34.0 ± 1.2	178.9 ± 4.8	3.4 ± 0.2	152.1 ± 3.3	10.5 ± 0.5*	8.0 ± 0.5
03, treatment	38.4 ± 3.3*	174.7 ± 4.7	3.4 ± 0.5	146.7 ± 3.4	9.9 ± 0.3*	7.9 ± 1.0
31, control	46.9 ± 3.5*	186.6 ± 2.7	2.9 ± 0.1*	153.1 ± 2.2	12.1 ± 0.3	9.8 ± 0.5
31, treatment	50.8 ± 4.2*	184.1 ± 2.1	3.0 ± 0.1	151.0 ± 1.4	11.7 ± 0.3	9.5 ± 0.6

Table 3. Summary of *P*-values from Mann-Whitney U tests to determine if there is a difference in blood plasma analytes between presence and absence of injury or feeding. Asterisks indicate a significant difference in the plasma analyte between the presence and absence of the indicator ($\alpha = 0.05$; with false discovery rate correction $P \leq 0.006$ in order to be significant at $\alpha = 0.05$).

Plasma analyte	Ruptured swimbladder	Ruptured Tunica exerna	Rete mirabile inflammation	Feeding
LDH	0.302	0.512	0.288	0.170
ALP	0.230	0.186	0.209	0.031
CK*	0.954	0.514	0.112	0.002*
AST	0.397	0.342	0.300	0.060
ALT	0.610	0.683	0.621	0.103
Glucose*	0.960	0.840	0.416	0.001*
Na	0.063	0.088	0.351	0.077
Cl	0.072	0.130	0.666	0.940
K	0.950	0.582	0.264	0.111
Ca*	0.016	0.057	0.011	0.000*
P*	0.395	0.119	0.102	0.005*
Cortisol*	0.036	0.061	0.002*	0.006*
IGF-1	0.056	0.137	0.667	0.105

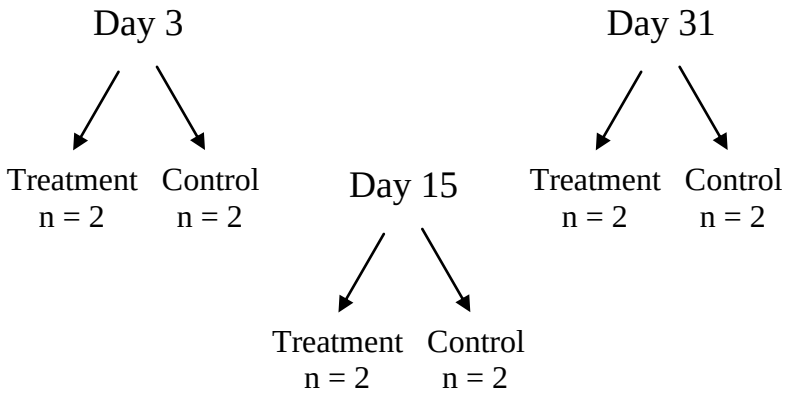
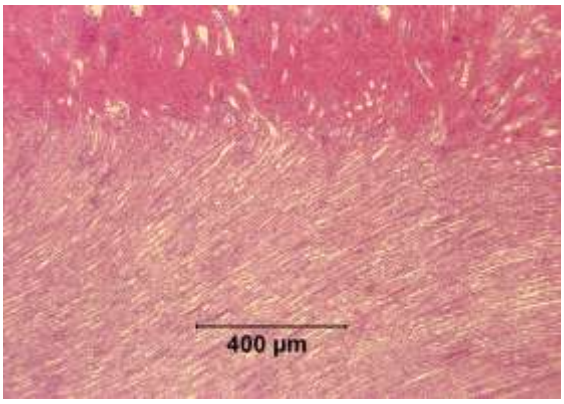


Figure 1. Experimental design of pressure chamber experiments. This experiment was replicated five times.

Figure 2. Histology of the rete mirabile. A) normal rete mirabile, B) chronic, multifocal inflammation; arrows indicate areas of inflammation, C) large emboli and hemorrhage associated with barotrauma.

A.



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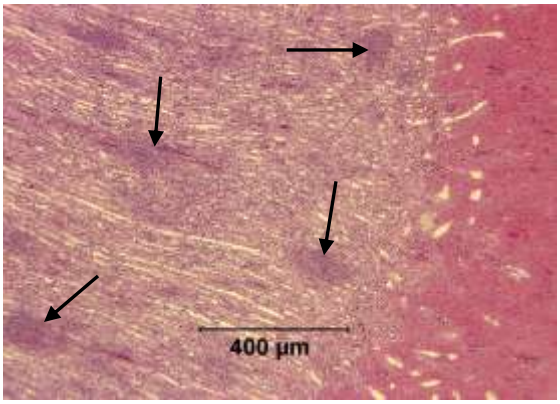

C.

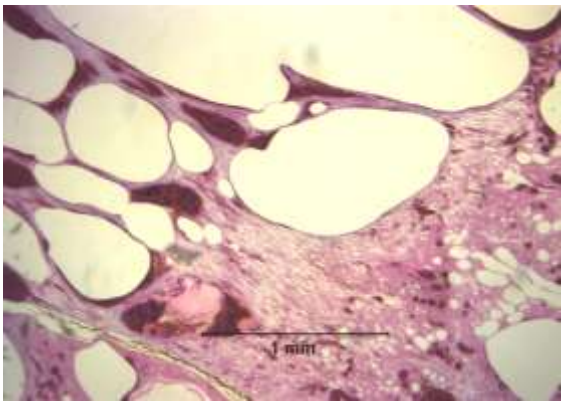

Figure 2.

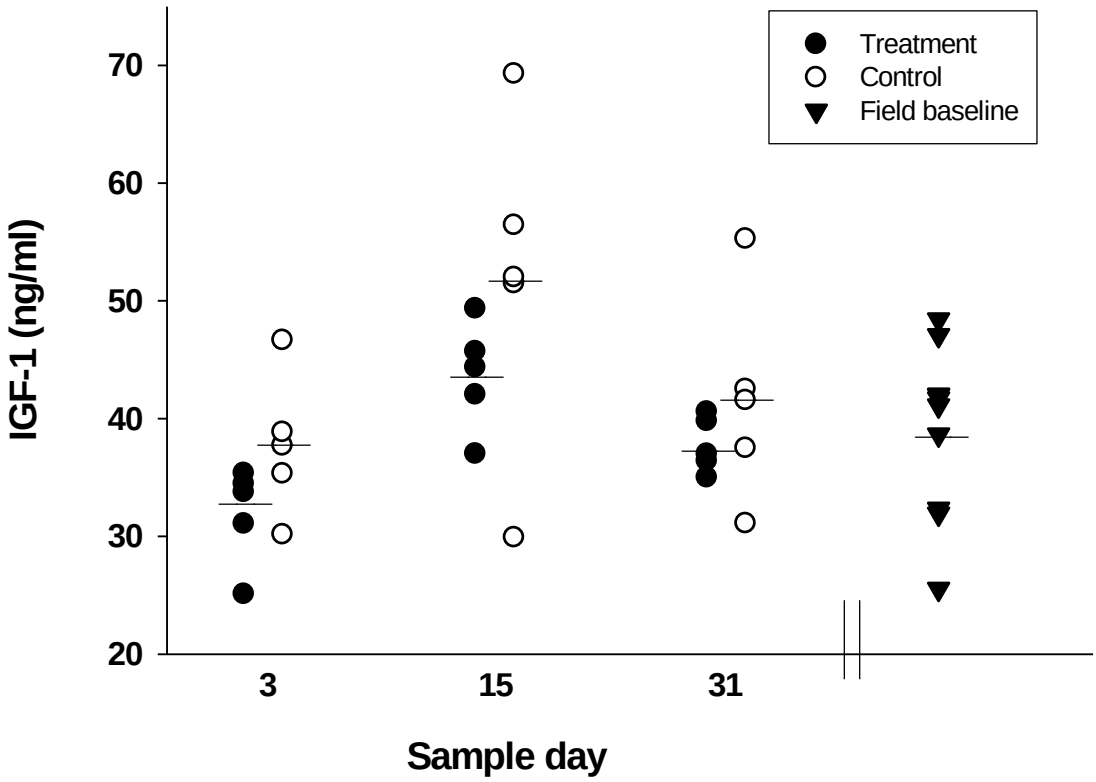

Figure 3. Individual IGF-1 values in treatment, control, and field baseline fish.

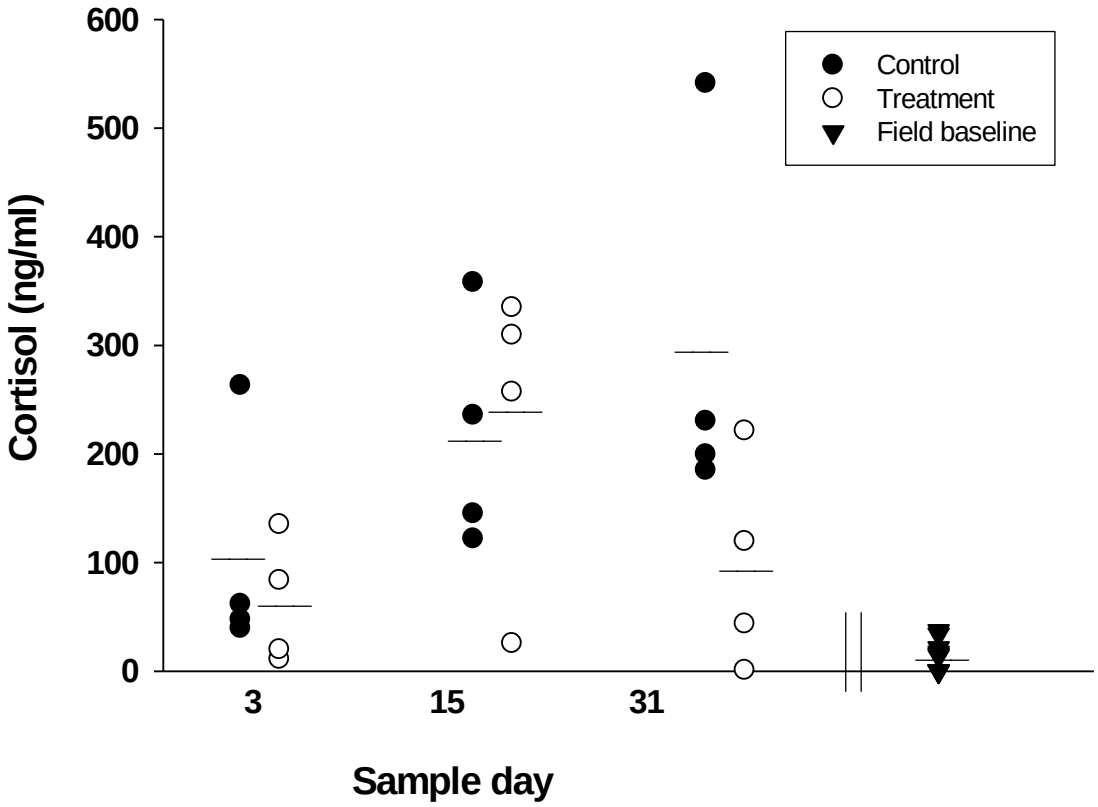

Figure 4. Individual cortisol values for treatment, control and field baseline fish.

Patterns of gene expression and recovery in Pacific rockfish following barotrauma with subsequent recompression

Alena L. Pribyl, Carl B. Schreck, Steven J. Parker, and Virginia M. Weis

Introduction

Recent advances in gene sequencing and the development of tools to analyze gene expression on a large scale have allowed studies of gene expression to expand to non-model organisms such as fish. In the last decade, custom microarrays have been developed for a variety of fish species such as African cichlid fish *Astatotilapia burtoni* (Renn et al. 2004), European flounder *Platichthys flesus* (Diab et al. 2008, Williams et al. 2003), annual killifish *Austrofundulus limnaeus* (Podrabsky and Somero 2004), rainbow trout *Oncorhynchus mykiss* (Krasnov et al. 2005), Atlantic salmon *Salmo salar* (Rise et al. 2004), carp *Cyprinus carpio* (Reynders et al. 2006) and largemouth bass *Micropterus salmoides* (Garcia-Reyero et al. 2008). The use of custom microarrays in fish has provided valuable insight into the molecular mechanisms that result from exposure to different environmental toxicants (Garcia-Reyero et al. 2008, Reynders et al. 2006, Williams et al. 2003), diseases (Diab et al. 2008, Kurobe et al. 2005), changing temperatures (Kassahn et al. 2007, Podrabsky and Somero 2004), and physical stressors (Krasnov et al. 2005). In this study, we designed a rockfish (*Sebastes*) specific cDNA microarray to gain insight into the molecular mechanisms governing pressure-related injuries incurred during capture in rockfish.

Genes involved in the response to capture in fish can be important to understand how capture affects fish and may provide insight into their ability to survive if released. This is especially important in depleted populations where discard of fish is required. The

rockfish genus is an important commercial and recreational fishery off the west coast of North America. Over 65 species occur between Baja California to Alaska, but since the 1980s, there has been a widespread decline in many rockfish populations (Love et al. 2002). Currently seven of these species have been declared depleted by the Pacific Fishery Management Council (PFMC 2008). Because of long generation times, late maturation, and sporadic recruitment, recovery of these populations is expected to take a long time. Bycatch of depleted rockfish is a common problem because different species of rockfish often have similar feeding habits and tend to aggregate in mixed-species assemblages. Current management tools for rockfish are limited to restricting fishing effort, location, and/or requiring discard of the incidentally caught species of concern. Discarding these species is required so they won't be targeted. Unfortunately, most rockfish species have very high mortality rates when discarded because of excessive buoyancy and pressure-related problems incurred during capture, referred to as barotrauma.

Barotrauma is a condition that commonly arises in physoclist fish, or fish that have a closed swimbladder, when they are captured and brought to the surface. When rockfish are captured and undergo a forced ascent, gas in the closed swimbladder expands as pressure is decreased (Boyle's Law), causing a variety of conditions collectively called barotrauma. Depending on the degree of pressure change, barotrauma in physoclist fish can involve bloating, ruptured swimbladder, crushed organs, eversion of the esophagus or

stomach, and exopthalmia (Gotshall 1964, Hannah et al. 2008a, Jarvis and Lowe 2008, Parker et al. 2006, Pribyl et al. 2009, Rummer and Bennett 2005). Research has been conducted on how barotrauma affects physoclist fish at the macroscopic and cellular level, but no work has been conducted on the genomic level.

Research has demonstrated that if excessively buoyant rockfish can be assisted in submerging quickly, these gases recompress, external barotrauma indicators disappear, and rockfish can survive in the short-term (Hannah and Matteson 2007, Jarvis and Lowe 2008). More recently, research on black rockfish has demonstrated that rockfish can survive for up to one month post-barotrauma, in the laboratory, when decompressed and recompressed from 35 m (Pribyl et al. 2010, *in preparation-b*). During this month-long recovery period rockfish resumed feeding, showed no changes in blood chemistry due to barotrauma (there were changes due to handling stress), and had no cellular injury in the heart, liver, head kidney, gonad, and eye that could be detected at the histological level. However, approximately 50 – 20% of rockfish sustained a ruptured swimbladder after the 31 day recovery period, and 7% of rockfish had a severely damaged rete mirabile (gas-concentrating organ in the swimbladder). It is unknown how these injuries will affect rockfish in the wild.

In order to gain a better understanding of the potential for black rockfish to recover from a forced decompression, our objective for this study was to analyze patterns of gene

expression in heart and liver tissue from the above experiment at three time points during the 31-day recovery period. If gene expression patterns between treatment and control rockfish show no differences during the recovery period, this would indicate that barotrauma does not impact black rockfish at the genetic level. If there are differences in patterns of gene expression between treatment and control fish, we can identify potential gene responsive to barotrauma and determine what pathways may be involved in the response. Little molecular work has been done to identify potential genes involved in injury and recovery in rockfish, thus in this study, we developed a custom cDNA microarray specific for black rockfish to identify potential candidate genes responsive to barotrauma. We then quantified gene expression levels of candidate genes using quantitative real-time PCR. By observing recovery from barotrauma at the molecular level, we can gain a better understanding of the recovery process in black rockfish and their potential for long-term survival.

Methods

Fish collection and recompression experiments

Collection and maintenance of black rockfish prior to the recompression experiments is described in Pribyl et al. (2010, *in preparation-b*). Recompression experiments are also described in Pribyl et al. (2010, *in preparation-b*), however briefly:

For each recompression experiment, six black rockfish were placed in each of two hyperbaric pressure chambers and adjusted to 4.5 ATA (approximately 35 m depth). One chamber served as a treatment chamber and the other chamber served as a control chamber. Once neutrally buoyant, fish in the treatment chamber were exposed to a simulated capture event by decreasing pressure to 1 ATA over a 90 sec period to induce decompression and barotrauma, held at surface pressure for 3 min, and then immediately recompressed to 4.5 ATA. Control fish remained at 4.5 ATA during this time. After a minimum recovery time of 6 hours post-recompression for treatment fish, both treatment and control fish were slowly brought to surface pressure with a 10% pressure reduction every 2-3 hours over a period of 3 days. Once at surface pressure, two rockfish from each chamber were immediately removed from the pressure chambers, examined for external barotrauma indicators, sampled for blood, euthanized, and dissected. Portions of liver and heart ventricle tissue were immediately removed and flash frozen in liquid nitrogen. Tissue samples were later stored at -80ºC until they could be processed. Two treatment and two control fish were subsequently sampled as described above at 15 days post-barotrauma and 31 days post-barotrauma. This experiment was replicated five times, for a total of 30 treatment fish and 30 control fish, or a total of 10 fish sampled per treatment and sample day.

Construction of cDNA microarray

Total RNA was extracted from liver and heart tissue using Trizol according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). All total RNA samples from the liver (n = 60) were pooled and mRNA was isolated from total RNA using Ambion's MicroPoly(A) kit (Applied Biosystems; Foster City, CA). The pooled liver mRNA from both treatment and control fish were used to create a liver cDNA library using Stratagene's Lambda ZAP-CMV XR library construction kit (Agilent Technologies; La Jolla, CA). We did not create a heart cDNA library due to insufficient total RNA yields from heart tissue. The pCMV-Script EX phagemid vector was mass excised from the Lambda Zap-CMV XR vector and the plasmid library was plated onto LB-kanamycin agar plates. A total of 5000 colonies were picked and grown in LB-kanamycin overnight in 384-well plates. These plates were replicated and replicate plates were supplemented with 10% glycerol and stored at -80ºC. The cDNA inserts were amplified with PCR using T3 forward and T7 reverse primers. We randomly checked approximately 5% of the amplified cDNA inserts with gel electrophoresis to ensure proper amplification and to determine the approximate size of cDNA inserts. The size of cDNA inserts varied between 0.4 and 2.0 Kb. We randomly selected 54 of the inserts (11%) and sequenced them to determine redundancy. Sequences were identified using Blastx. Of the 54 sequences, 13 represented vector (24%), 6 represented unknown genes (11%), and 35 (65%) represented known genes. Of the known genes, 16 genes (46%) were represented once and 5 genes (14%) were represented multiple times. The most abundant genes were ribosomal genes (19%) and apolipoproteins (19%).

The PCR-amplified cDNA was buffered in 3x saline-sodium citrate (SSC) and 1.5 M betaine and spotted on Corning UltraGAPS coated slides in two replicate blocks (each block contained 16, 18 x 18 spot "minor" blocks). Slides were printed at Oregon State University's Center for Genome Research and Biocomputing (CGRB) Core lab using a MicroGrid II (Digilab; Holliston, MA). Once printed, the array slides were dried for 48 h in a vacuum desiccator and UV cross linked at 300 mJ. Arrays were then stored in a vacuum desiccator until hybridization.

Hybridization of cDNA microarray

We constructed probes for hybridization by pooling total RNA according to tissue, treatment group, and sample day for a total of twelve "experimental" pools of mRNA (Figure 1). Another "reference" pool of mRNA was created by combining total RNA from all liver and heart samples (n = 120).

For probe synthesis and hybridization we used Genisphere's Array 900 microarray kit and used the manufacturer's protocol. We synthesized cDNA for each probe from 0.7 μg of total RNA. All array slides were prewashed and prehybridized prior to hybridization with cDNA. Once array slides were ready, LifterSlips were applied to each array and experimental and reference cDNA in enhanced cDNA hybridization buffer was applied to each slide and incubated overnight at 55ºC in sealed hybridization chambers. The

following day, after post-hybridization washes, we again applied a LifterSlip to each array and hybridized the arrays with Cy3 and Cy5 3DNA Capture Reagents in an SDS-based hybridization buffer at 55ºC in sealed hybridization chambers. After hybridization, slides were washed and dried according to the manufacturer's instructions, and immediately scanned on an Axon GenePix 4200A scanner. The software GenePix Pro 5 was used to align slides, apply quality control to individual spots, and extract data from all slides.

Microarray experimental design and statistical analyses

For this experiment we utilized a reference design with a dye swap to determine changes in gene expression between treatment and control pools within days 3, 15 and 31 post-barotrauma. Each experimental pool (Figure 1) was compared to the reference pool for a total of 12 arrays hybridized for each experiment, or 24 arrays hybridized in total, including the dye swap. Fluorescence intensity values were normalized based on a ratio of medians relative to 32 control spots placed in each 18 spot x 18 spot minor block. Control spots consisted of a pool of all 120 samples, thus it was assumed experimental and reference samples would bind to these spots equally; the ratio of medians for these features was set to 1.0. To determine changes in gene expression between treatment and control pools within each sample day, we averaged normalized intensity values from the two blocks on each slide, and determined the Cy3/Cy5 ratio representing the treatment pool relative to the reference pool (Cy5/Cy3 for the dye swap). We then normalized the

treatment ratio relative to the control ratio to determine the fold change in gene expression between treatment and control pools. To determine genes for sequencing, we searched for ratios greater than 2.0 in both dye swaps from day 3 post-barotrauma. We chose to pool our samples for the microarray because our main purpose for the microarray was to identify candidate genes, and not to quantify their expression with the microarray. This meant however, that we had no degrees of freedom and thus could not run statistical analyses on the microarray data. All analyses were conducted on Acuity 4.0.

Sequencing

We sequenced differentially expressed cDNA inserts using the T3 forward primer. Sequencing was conducted at Oregon State University's CGRB Core lab using an ABI Prism 3730 Genetic Analyzer. We manually removed vector fragments from the sequence and, if necessary, removed low quality sections of the sequence. Sequences were then identified by homology to known sequences using a Blastx search of the GenBank database. All identified sequences had an expected value of less than 1×10^{-6} and thus were considered homologs to our sequence.

Quantitative real time PCR

We chose several genes of interest that were shown to be differentially regulated by our microarray data and designed primers for our genes of interest using Primer3 that

amplified 150 – 200 bp fragments. All primers were checked for specific amplification of appropriately sized bands using PCR and gel electrophoresis prior to use in real time PCR reactions.

For both liver and heart tissues, we pooled total RNA from the two pseudoreplicates within each experiment for a total of 30 pools per tissue. This gave us five biological replicates per sample day and treatment group. We synthesized cDNA from pseudoreplicate pools of total RNA using Invitrogen's SuperScript III First Strand Synthesis System for RT-PCR according to the manufacturer's instructions. We synthesized cDNA using 5.0 µg of total RNA for liver samples and 1.8 µg of total RNA for heart samples. Liver samples were diluted 1:100 and heart samples were diluted 1:30. Quantitative real-time PCR (QRT-PCR) reactions were carried out using 4 µl of cDNA in a 20 µl reaction with 5 µM primers and SYBR Premix Ex Taq (Takara Bio Inc; Otsu, Shiga, Japan) according to the manufacturer's instructions. All samples were measured in triplicate with no-template controls using an ABI PRISM 7400 Fast Real-Time PCR System for a total of 40 cycles (Figure 2). A melting disassociation curve was run after each reaction to confirm the amplification of specific product only.

We used the comparative CT method corrected for actual PCR efficiency to determine relative quantities of mRNA transcripts in each cDNA sample. Actual PCR efficiencies were determined using LinRegPCR (Ramakers et al. 2003, Ruijter et al. 2009). We

normalized QRT-PCR expression data with two housekeeping genes; 60S ribosomal protein L13 and ubiquitin. We identified 60S ribosomal protein L13 from our microarray, and we found ubiquitin by testing several potential housekeeping genes for fish from a literature search, including acidic ribosomal protein, ribosomal protein L37 (Olsvik et al. 2008), and ubiquitin (Geist et al. 2007, Olsvik et al. 2008). Ubiquitin was the only gene with enough conserved sequence where we could develop a primer that worked in rockfish. Expression stability of housekeeping genes was analyzed using geNorm (Vandesompele et al. 2002) and was 0.116 for both genes. We used the normalization factor calculated by geNorm to normalize each cDNA sample.

QRT-PCR statistical analyses

We used the nonparametric Mann-Whitney test to identify expression differences between treatment and control fish within each sample day. Because we conducted multiple tests on data from the same experimental unit we used a false discovery rate correction as described in Waite and Campbell (2006).

We also used Mann-Whitney tests to determine if there was a difference in gene expression levels between the presence and absence of injury or feeding in the fish. Presence/absence injuries included: ruptured swimbladder and rete mirabile inflammation. Injuries and feeding were observed in these fish in a previous study (Pribyl et al. 2010, *in preparation-b*). We applied a false discovery rate correction to these *P*-

values. We also tested to see if there was a difference in gene expression values between different parasite loads in the heart, liver and head kidney. We analyzed the parasite loads from histology slides (Pribyl et al. 2010, *in preparation-a*) and characterized each tissue as having no infection, a light infection (three or fewer parasites in a section), or a heavy infection (more than three parasites in a section). We then averaged the values for pseudoreplicates and used a Kruskal-Wallace test to determine if there were differences in gene expression between each category. All statistical analyses were conducted with SPSS v.17.0.

Results

Microarray

For the liver microarray hybridizations, we found a total of 44 genes up-regulated more than two-fold and 15 genes down-regulated more than two-fold relative to our control samples for day 3. These genes were no longer differentially regulated at days 15 and 31. We chose to sequence approximately half of the differentially expressed genes from day 3. We randomly chose 19 of the up-regulated and 10 of the down-regulated genes for sequencing. For the heart microarray hybridizations, we found a total of 14 genes up-regulated more than 1.5-fold (no genes were up-regulated more than two-fold), and 12 genes down-regulated more than two-fold relative to our control samples for day 3. We randomly chose 7 of the up-regulated genes and 6 of the down-regulated genes for sequencing.

Of the 29 genes we sequenced from the liver (Table 1), two contained no inserts, seven were represented multiple times, and seven were represented once. From the 13 genes sequenced for the heart (Table 1), two contained no inserts, three were represented multiple times, and two were represented once. We chose nine genes of interest in the liver and four genes of interest in the heart for validation with QRT-PCR (Table 2).

QRT-PCR

The QRT-PCR results generally supported the trends we observed from the microarray analyses for up-regulated genes in the liver (Table 3); however genes identified from the microarray that were down-regulated in the liver (Table 3) and differentially regulated in the heart (Table 4) did not support the trends we observed from microarray analyses. Genes from the microarray that were down-regulated in the liver and genes that were differentially regulated in the heart at day 3 all showed a high level of variation between biological replicates when checked with QRT-PCR. Some replicates were up-regulated while other replicates were down-regulated within the same gene. All genes from the microarray that showed up-regulation in the liver at day 3, however, were consistently up-regulated at the biological replicate level and showed significant differences between treatment and control groups (Table 3). By days 3 and 15, these same genes showed a trend towards returning to control levels by being neutrally expressed (Figure 3). The up-regulated genes included complement C1q-like protein 2, complement component C3,

complement regulatory plasma protein, serum amyloid A-5, c-type lysozyme, and hepcidin precursor type I. None of these genes were significantly different between treatment and control groups by day 31. Data from treatment:control ratios of C1q-like protein 2, complement component C3, complement regulatory plasma protein, and hepcidin over time also showed a good fit to an inverse first order curve, illustrating the return to neutral regulation.

Modeling expression values

Complement component C1q-like protein 2 had higher expression levels in fish that had a ruptured swimbladder compared to fish that did not have a ruptured swimbladder (Mann-Whitney test, $P = 0.008$). We found no other statistical differences in gene expression values and presence/absence of a ruptured swimbladder, rete mirabile inflammation, or feeding after correcting for false discovery rates.

We identified parasites in the heart, liver and head kidney of rockfish. No parasites were observed in the eye, rete mirabile or gonad. In the heart atrium and ventricle, several fish had light to heavy infections of blood flukes (both adults and eggs). In the head kidney, several fish had light to heavy infections of *Ichthyophonous* sp. and granulomas possibly indicative of a bacterial infection. In the liver, a large proportion of rockfish had light to heavy infections by a myxozoan parasite, and a couple rockfish had encysted tapeworms and larval nematodes. We found no differences in gene expression levels of the six up-

regulated liver genes among the three categories of parasite infection in the heart (Kruskal-Wallis test, df = 2; $P > 0.50$ for all six tests), head kidney (Kruskal-Wallis test, df = 2; $P > 0.20$ for all six tests), or liver (Kruskal-Wallis test; df = 2; $P > 0.15$ for all six tests).

Discussion

We found six genes responsive to barotrauma in black rockfish utilizing our rockfish-specific microarray: complement C1q-like protein 2, complement component C3, complement regulatory plasma protein, serum amyloid A-5, c-type lysozyme, and hepcidin precursor type I. All of these genes were significantly elevated in treatment rockfish compared to control rockfish at day 3 post-barotrauma, even after accounting for the handling stress both treatment and control fish experienced during the experiments. These genes were no longer elevated by days 15 and 31 post-barotrauma, indicating probable recovery in treatment rockfish over time from barotrauma.

Each of the six genes we found is associated with the innate immune system. The innate immune system is non-specific and the first line of defense against pathogens. In mammals, the complement system is one of the central immune responses initiated by the innate immune system. The complement system consists of about 20 proteins that are part of a biochemical cascade when activated, and can recruit inflammatory cells, opsonize pathogens, and kill pathogens (Janeway et al. 2005). Three of the genes

responsive to barotrauma are part of the complement system: Complement C1q, complement component C3, and complement regulatory plasma protein. Complement C1q initiates the classical pathway of complement activation by binding to antigen:antibody complexes and to pathogen surfaces. Complement component C3 initiates the activation of the alternative pathway of complement activation by binding to pathogen surfaces. In addition, all three complement pathways produce C3 convertase, which cleaves complement component C3 into C3a and C3b. C3a recruits inflammatory cells to the site of infection and C3b binds to bacterial cell membranes and opsonizes the bacteria (Janeway et al. 2005). Thus complement component C3 plays a primary role in the activation of the complement system. Complement regulatory plasma proteins regulate complement activation and protect the host against cell damage from the complement system (Meri and Jarva 2008). Regulation of the complement system in fish in response to pathogen and toxicant stress appears to be variable, depending on the stressor and species. The complement system was down-regulated in rainbow trout *Oncorhynchus mykiss* exposed to *Yersinia ruckeri* (Raida and Buchmann 2009), however it was up-regulated in rainbow trout exposed to *Vibrio anguillarum* (Bayne et al. 2001) and in carp *Cyprinus carpio* exposed to cadmium (Reynders et al. 2006). Raida and Buchmann (2009) suggested that one possibility for the down-regulation of the complement response was that the level of infection was insufficient to initiate activation of the complement system. In our study, it appears barotrauma is a severe enough stressor to activate the complement system.

Serum amyloid A-5 is part of the serum amyloid A (SAA) superfamily of acute phase proteins which are primarily produced by the liver (although other organs have also been reported as producing SAAs in response to inflammation (Villarroel et al. 2008). In fish, SAA is a common acute phase protein that is activated in response to a variety of stressors (Jensen et al. 1997, Jensen and Whitehead 1998, Raida and Buchmann 2009, Talbot et al. 2009). Levels of SAA mRNA showed over a 40-fold increase in Arctic charr *Salvelinus alpinus* challenged with *Aeromonas salmonicida* after 5 days (Jensen et al. 1997). Levels of SAA mRNA peaked at a 3000-fold increase in rainbow trout challenged with *Yersinia ruckeri* after 3 days, remained elevated after 14 days, but returned close to control levels after 28 days (Raida and Buchmann 2009). Expression levels of SAA in rainbow trout subjected to confinement stress was highly variable, with three fish showing elevated levels (18 to 221-fold increases) of SAA after 7 days of confinement and two fish showing no increase. These studies suggest SAA levels do not peak immediately after a stressor, but take a few days to reach peak levels. Thus, the peak in SAA expression levels in two of the replicates 15 days post-barotrauma may not be unusual; it is also possible SAA levels peaked earlier than 15 days post-barotrauma and most fish had already returned to neutral regulation levels, while other fish were taking longer to return to neutral regulation. The decline towards neutral expression at day 31 in all treatment fish is a good indicator that fish were recovering from barotrauma.

Lysozymes are important antimicrobial agents of the innate immune system, and are produced in the liver as well as other tissues. Besides having antimicrobial properties, lysozymes can also activate the complement system or act as opsonins (molecules that enhance the binding of a phagocyte to the antigen). The c-type lysozyme is thus named because the lysozyme was originally obtained from chickens (chicken-type lysozyme) (Saurabh and Sahoo 2008). Lysozyme activity in fish, similar to complement, also appears to vary depending on the stressor. Stressors such as transport (Möck and Peters 1990), water pollution (Möck and Peters 1990), and subordination stress (Caruso and Lazard 1999) caused a reduction in lysozyme levels whereas other stressors such as handling stress (Caruso et al. 2002) and disease infection (Demers and Bayne 1997) caused an increase in lysozyme levels. Again, barotrauma appears to be a sufficient stressor to cause an increase in lysozyme levels, however after 31 days of recovery lysozyme levels declined, again indicating possible recovery.

The final gene we confirmed with QRT-PCR was hepcidin. Hepcidin is a recently discovered protein (Park et al. 2001) and is responsible for regulating plasma iron concentrations and for controlling the distribution of iron to tissues in order to maintain homeostasis (Nemeth and Ganz 2006). Hepcidin is also associated with the innate immune system as an antimicrobial peptide because it becomes elevated during infection and inflammation. It is likely that hepcidin plays a role in limiting iron availability to foreign invaders (Nemeth and Ganz 2006). Rainbow trout exposed to *V. anguillarum*

showed up-regulation of hepcidin by 22-fold (Gerwick et al. 2007) and European flounder *Platichthys flesus* exposed to *A. salmonicida* showed up-regulation of hepcidin by over 6-fold (Diab et al. 2008). Korean black rockfish *Sebastes schlegelii* also showed up-regulation of two different types of hepcidin when infected with *Streptococcus iniae*. (Kim et al. 2008). In our study, hepcidin was up-regulated an average of 20.4-fold in rockfish 3 days post-barotrauma but dropped to almost neutral expression after 31 days recovery.

Modeling of the six up-regulated genes against the presence and absence of injury and feeding previously observed in these rockfish, showed complement C1q had higher expression levels in fish with a ruptured swimbladder compared to fish without a ruptured swimbladder. Between 20% – 50% of treatment fish could not hold gas in their swimbladder after 31-days post-barotrauma (Pribyl et al. 2010, *in preparation-b*). Fish with a ruptured swimbladder will not be able to maintain neutral buoyancy in the water column, and thus may need to expend more energy to find and capture prey, as well as to escape predation. In addition, rockfish use their swimbladder for communication (Hallacher 1974), thus a ruptured swimbladder may have social ramifications as well. It is unclear how this will affect a fish's overall fitness, although survival will likely be dependent on ocean conditions at the time of release. Elevated complement C1q in fish with a ruptured swimbladder indicates a ruptured swimbladder may also affect the

complement system in fish. This suggests that the return to neutral regulation in complement C1q may be a good indicator of recovery as well as injury in rockfish.

We define recovery in rockfish as a return to control conditions, however it is important to acknowledge that our control fish were under laboratory conditions for several months and experienced netting, transport, confinement, and a two-week fasting period during the course of the experiments. A better evaluation of recovery would be to compare our treatment rockfish to a field baseline from the wild, however it is very difficult to obtain a true field baseline sample because rockfish would still need to be brought to the surface for sampling, and we would not know from what depth rockfish were neutrally buoyant and if some genes were already responding as a result of the barotrauma and capture stress. In addition, variability in ocean conditions could affect the gene profile of the field baseline, making it difficult to determine a "normal" profile. By keeping all rockfish at the same conditions one month prior to experiments, and utilizing hyperbaric pressure chambers to ensure all fish were acclimated to the same depth, we could make certain the only difference between our treatment and control fish was induced decompression resulting in barotrauma and subsequent recompression.

The up-regulation of six genes from the innate immune system strongly suggests that the innate immune system was activated in response to barotrauma and not as a result of handling stress. This is a novel discovery, because as seen in our physiological analysis

of these fish (Pribyl et al. 2010, *in preparation-b*), the handling stress that both treatment and control groups experienced outweighed the additional stress of barotrauma for many typical measures of stress (ie: cortisol, glucose). Finding a difference at the gene expression level indicates that barotrauma does affect fish to a greater degree than other handling stressors. We likely did not see more genes differentially regulated in our microarray because of the variety of stressors incurred by our fish, and the high level of individual variation among fish in response to stress. This may also be why we observed the high level of variation among differentially regulated genes in the heart and down-regulated genes in the liver. The return to neutral regulation of the six up-regulated genes in most fish by days 15 and 31 suggests fish were recovering from barotrauma, however because we did not have a true field baseline, we cannot say these fish were completely recovered. Other genes associated with the immune system in response to handling stress may still have been up-regulated, but we would not have been able to detect these due to the handling stress our control group also experienced. In conclusion, results from this study add support to the conclusion that black rockfish decompressed from 35 m and subsequently recompressed have a high potential for recovery from barotrauma in laboratory conditions.

Acknowledgements

We would like to thank Caprice Rosato at the Center for Genome Research and Biocomputing at Oregon State University for printing the arrays, instruction on

equipment usage, and advice on molecular techniques. We thank Wendy Phillips, Christy Schnitzler and Tracey Momoda for assistance with molecular techniques and advice on analyses. This research was funded by a NOAA Saltonstall-Kennedy grant, a Mamie Markham Research Award (Hatfield Marine Science Center, Oregon State University), the Department of Fisheries and Wildlife at Oregon State University, and the Oregon Cooperative Fisheries Research Unit at Oregon State University.

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Table 1. Differentially regulated genes that were sequenced and identified from the rockfish-specific cDNA microarray in heart and liver tissue. Reps is the number of times each gene each gene was represented. Direction refers to the direction of differential regulation. Genes were considered differentially regulated if the fold change was greater than 2, except for up-regulation in the heart where fold change was greater than 1.5.

Gene	Genbank Accession ID	dbEST ID	Reps	Direction	Tissue
Hepcidin precursor type I	GT617666	67785081	4	Up	Liver
Serum amyloid A-5	GT617654	67785069	2	Up	Liver
C-type lysozyme	GT617660	67785075	4	Up	Liver
Complement regulatory plasma protein	GT617661	67785076	3	Up	Liver
MID1 interacting-like protein	GT617659	67785074	3	Down	Liver
Glyceraldehyde-3-phosphate dehydrogenase	GT617674	67785089	2	Down	Liver
Fructose-bisphosphate aldolase B	GT617672	67785087	2	Down	Liver
Complement C1q-like protein 2	GT617675	67785090	1	Up	Liver
Complement component C3	GT617665	67785080	1	Up	Liver
Warm temp acclimation related-like 65kDa protein	GT617671	67785086	1	Up	Liver
NADH dehydrogenase subunit 5	GW603858	68981602	1	Up	Liver
Haptoglobin	GW603857	68981601	1	Up	Liver
Inter-alpha (globulin) inhibitor H4 isoform 1	GT617669	67785084	1	Up	Liver
Chemokine CC	GT617657	67785072	1	Up	Heart
Type II antifreeze protein	GT617658	67785073	2	Up	Heart
Apolipoprotein A-1	GT617653	67785068	4	Down	Heart
14kd Apolipoprotein	GT617655	67785070	2	Down	Heart
60S ribosomal protein L8	GT617670	67785085	1	Up	Heart
Gamma-glutamyl hydrolase precursor	GT617667	67785082	1	Up	Heart

Table 2. Forward and reverse primers for genes of interest used in QRT-PCR reactions. * = housekeeping genes

Gene	Primer sequence
Complement c1q-like protein 2	Fwd: TCTGCTGACCCTAAGCCTGT Rev: ACAGAGAAGGCCACTTTGGA
Complement component c3	Fwd: CGGAGGCTATGGATCAACTC Rev: GATGTTCTGGTGGCGTAGTG
Complement regulatory plasma protein	Fwd: ATGGTGAATGGGTTGGAGAG Rev: ATCGTGTCTTCGTCCACCTT
C-type lysozyme	Fwd: TGGAACCTCCTTATCGAACG Rev: ATCCTGCCACATAGGAGCTG
Serum amyloid A-5	Fwd: ATGATATGAGGGACGCCAAC Rev: ATGCTCATTTGCCCTCTGAT
Hepcidin precursor type I	Fwd: CCGTCCGAAGATGAAGACAT Rev: TCAGCAGCAACTGGATTGTC
MID-1 interacting-like protein	Fwd: ACAGCCAACCACATCCAACT Rev: TCACAGTCCCACATCTCATCA
Fructose bisphosphate aldolase	Fwd: CGTGACCTCCTCTTCTCCAC Rev: GTGCCTTTGTCCACCTTGAT
Gylceraldeyhde-3-phosphate dehydrogenase	Fwd: CCAGGTCGTCTCCACAGACT Rev: GCGGGTCAGTTTACTCCTTG
14 kD apolipoprotein	Fwd: AAGATCAACTGGAGCGAAGG Rev: TTTGCATAGGTGGTGTTCG
Apolipoprotein A-1	Fwd: CAGAGCATCAACACCGATGA Rev: TATGGCTGCGTATGTGAGGA
Type II antifreeze protein	Fwd: ACGAGGAGACCACGGATCAT Rev: GGCACTTCTGGTGCTTGACT
CC chemokine	Fwd: ATGAGCCACCGCTATGATTC Rev: GCGTACCAACACACTCTTGC
60S ribosomal protein L13*	Fwd: TGGAACCTCCTTATCGAACG Rev: ATCCTGCCACATAGGAGCTG
Ubiquitin*	Fwd: TGAGCCCAGTGACACTATCG Rev: GAGAGAAGGCTCGATGATGC

Table 3. Genes of interest from liver hybridizations that were checked using QRT-PCR. Ratios for QRT-PCR are averages of the five biological replicates with the standard error in parentheses. * = difference at $\alpha = 0.05$ level between treatment and control samples (Mann-Whitney test with false discovery rate correction).

Gene (function)	Genbank Accession ID	dbEST ID	Sample day	Ratio of treatment : control	
				Microarray[a]	QRT-PCR
Complement c1q-like protein 2 (innate immune system)	GT617675	67785090	3	4.94	20.27 (±3.30)*
			15	1.47	6.51 (±2.11)
			31	1.07	2.37 (±0.34)
Complement component c3 (innate immune system)	GT617665	67785080	3	2.27	3.81 (±0.77)*
			15	1.06	1.61 (±0.42)
			31	1.25	1.05 (±0.44)
Complement regulatory plasma protein (innate immune system)	GT617661	67785076	3	2.36	2.90 (±0.28)*
			15	1.29	1.37 (±0.34)
			31	1.11	1.32 (±0.10)
C-type lysozyme (innate immune system)	GT617660	67785075	3	2.48	5.49 (±1.93)*
			15	1.31	5.73 (±4.32)
			31	1.29	1.19 (±0.74)
Serum amyloid A-5 protein (innate immune system)	GT617654	67785069	3	3.12	6.94 (±2.23)*
			15	4.93	26.64 (±17.20)
			31	1.66	1.56 (±0.66)
Hepcidin precursor type I (iron regulation/innate immune system)	GT617666	67785081	3	3.76	20.42 (±9.25)*
			15	1.25	2.50 (±1.64)
			31	1.32	1.46 (±0.68)
MID-1 interacting-like protein (microtubule stabilization; cell division)	GT617659	67785074	3	0.45	0.48 (±0.32)
			15	1.29	2.42 (±1.16)
			31	0.87	1.22 (±0.53)
Fructose bisphosphate aldolase (glycolysis)	GT617672	67785087	3	0.47	1.16 (±0.58)
			15	1.44	1.23 (±0.60)
			31	0.88	1.02 (±0.53)
Glyceraldeyhde-3-phosphate dehydrogenase (glycolysis)	GT617674	67785089	3	0.42	0.76 (±0.18)
			15	0.90	1.13 (± 0.27)
			31	0.72	0.88 (±0.43)

[a] Ratio of treatment : control for microarray = (mean values of treatment pool/reference pool) / (mean values of control pool/reference pool); for QRT-PCR = (normalized treatment sample) / (normalized control sample)

Table 4. Genes of interest from heart ventricle hybridizations that were checked using QRT-PCR. Ratios for QRT-PCR are averages of the five biological replicates with the standard error in parentheses.

Gene	Genbank Accession ID	dbEST ID	Sample day	Ratio of treatment:control	
				Microarray[a]	QRT-PCR
14 kD apolipoprotein (biological function unknown)	GT617655	67785070	3	0.44	1.29 (±0.41)
			15	0.63	1.07 (±0.29)
			31	1.31	2.47 (±1.40)
Apolipoprotein A-1 (cholesterol/lipid metabolism, transport)	GT617653	67785068	3	0.42	0.71 (±0.17)
			15	0.57	1.91 (±1.00)
			31	1.89	1.76 (±0.80)
Type II antifreeze protein (lowers freezing temp of blood)	GT617658	67785073	3	2.28	3.88 (±2.42)
			15	1.11	6.18 (±3.81)
			31	0.82	2.27 (±0.99)
Chemokine CC-like protein (innate immune system)	GT617657	67785072	3	1.54	1.44 (±0.47)
			15	2.07	2.38 (±0.65)
			31	0.78	1.38 (±0.35)

[a] Ratio of treatment : control for microarray = (mean values of treatment pool/reference pool) / (mean values of control pool/reference pool); for QRT-PCR = (normalized treatment samples) / (normalized control samples)

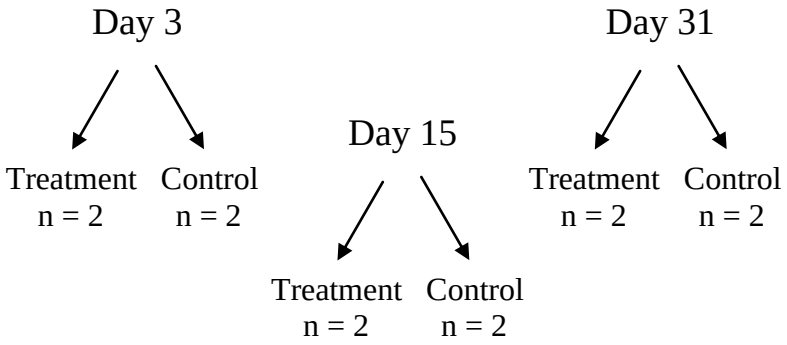

Figure 1. The experimental pools used to create probes for microarray hybridizations. We pooled total RNA from liver and heart in the 6 pools described above, for a total of 12 probes. Figure is taken from Pribyl et al. (2010, *in preparation-b*).

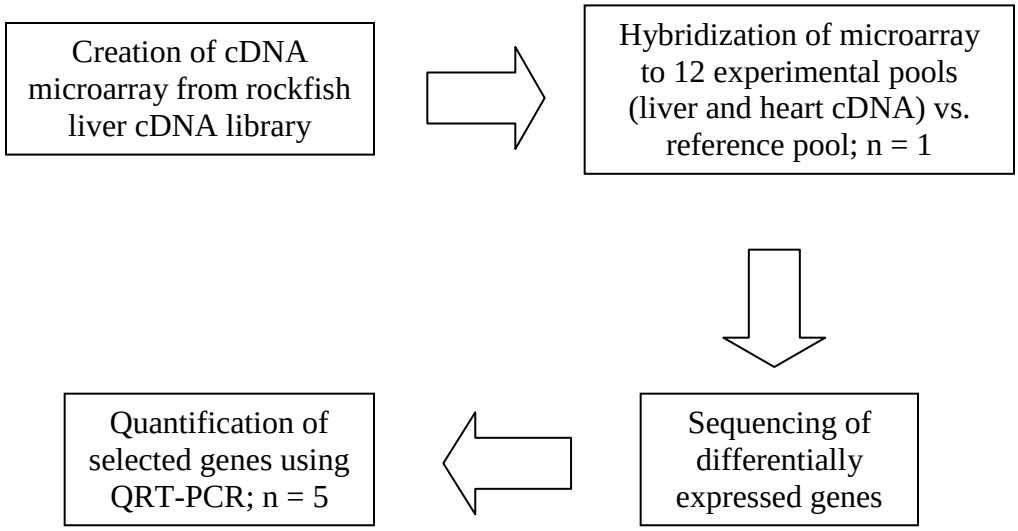

Figure 2. Flowchart of analyses leading up to the final quantification of differentially expressed genes in treatment relative to control fish within each sample day. N is the number of biological replicates.

Figure 3. Gene expression of biological replicates at days 3, 15, and 31 post-barotrauma for up-regulated genes in the liver. Each circle represents averaged pseudoreplicates from two fish. N = 5 for each sample day (some circles overlap and thus are difficult to differentiate). X-axis is set at y = 1 to indicate neutral gene regulation.

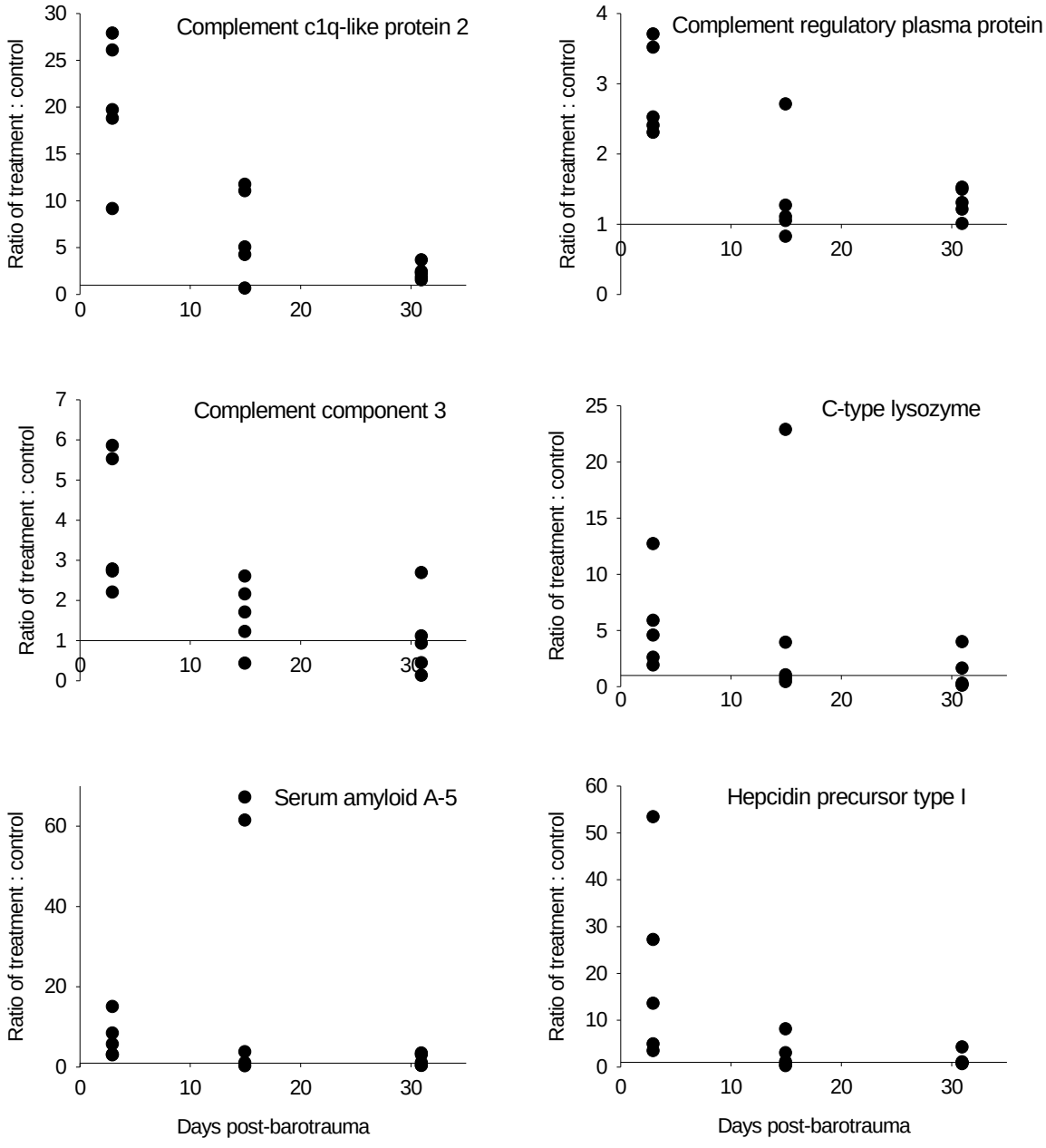
Complement c1q-like protein 2
Complement regulatory plasma protein
Complement component 3
C-type lysozyme
Serum amyloid A-5
Hepcidin precursor type I
Ratio of treatment : control
Days post-barotrauma

Figure 3.

General Conclusions

Response to Decompression

A variety of macroscopic and microscopic changes occurred in rockfish as a result of rapid decompression and barotrauma. We found many of these changes differed between species and depth of capture.

Macroscopic injuries associated with barotrauma were often species-specific. For example, yellowtail rockfish and quillback rockfish had fewer macroscopic barotrauma injuries than other species we sampled. Although quillback rockfish tend to be bottom dwellers and yellowtail rockfish are semi-pelagic, both yellowtail rockfish and quillback rockfish have been observed releasing gas bubbles from their pharyngo-cleithral membrane during forced decompression (Hannah et al. 2008a, Pearcy 1992). When Pearcy (1992) made this observation in yellowtail rockfish, he found the gas to be of the same composition as gas in the swimbladder. Hannah et al. (2008a) provided further evidence that species such as yellowtail rockfish and quillback rockfish are releasing excess gas from their swimbladder through the pharyngo-cleithral membrane by showing that during decompression, eversion of the esophagus and exophthalmia in rockfish is caused by gas escaping from an unruptured or ruptured swimbladder and moving in an anterio-dorsal direction. Because yellowtail and quillback rockfish are able to release excess gas from the swimbladder through leakages in the swimbladder wall and in the pharyngo-cleithral membrane, trapped gas likely does not build up enough pressure to

cause macroscopic indicators of barotrauma, such as everted esophagus, exophthalmia and ruptured swimbladders (Hannah et al. 2008b). It is unclear if the presence of leaky membranes in these two rockfish species is an evolutionary adaptation or just coincidence. An argument for adaptation could be used for yellowtail rockfish because they are known to make rapid vertical "bounce" dives ranging up to 35 m (Pearcy 1992), which could necessitate a mechanism to deal with expanding swimbladder gases. Quillback rockfish, however, are unknown to make rapid vertical ascents, or to even move more than a few vertical meters in the water column at a time, thus it is unclear why a leaky membrane would be of benefit to a quillback rockfish.

Depth of capture was the second factor that played an important role in the presence of macroscopic barotrauma indicators. The presence of exophthalmia, ocular emphysema, and a ruptured swimbladder were all influenced by depth of capture. Studies on rockfish (Hannah et al. 2008a, Jarvis and Lowe 2008) and other fish species such as red snapper *Lutjanus campechanus* (Gitschlag and Renaud 1994, Rummer and Bennett 2005), largemouthmouth bass *Micropterus salmoides* (Feathers and Knable 1983), Australasian snapper *Pagrus auratus* (Longbottom 2000), and West Australian dhufish *Glaucosoma hebraicum* (St John and Syers 2005), also show an increase in the number of barotrauma indicators with depth of capture. Depth of capture was also shown to increase behavioral impairment in black, blue and yelloweye rockfish when released from a cage after capture and recompression (Hannah and Matteson 2007).

Microscopically, we also found an increasing number of barotrauma-related injuries with depth of capture. When rockfish were decompressed from a simulated 35 m depth, the only tissue-level injury from barotrauma we observed was emphysema in the heart ventricle. There was no injury in the liver, head kidney, gill, or pseudobranch. When rockfish were captured in the field at depths exceeding 35 m, we found a low proportion of fish with emboli in the vessels of the head kidney and rete mirabile in addition to emphysema in the heart ventricle. We could not detect a difference between the presence of these histologic barotrauma indicators among species; because of small sample sizes it is unclear if there is truly no interspecific effect or if the sample size was not large enough to show a difference among species. Emphysema was present in the compact myocardium of the heart ventricle in a small proportion of all rockfish species except for yellowtail rockfish. Longbottom (2000) also observed lesions in the heart ventricle of the Australasian snapper *Pagrus auratus* after capture from depths ranging between 10 – 35 m, and D'Aoust and Smith (1974) observed histological evidence of gas bubbles in the somatic muscle tissue of fingerling rainbow trout *Oncorhynchus mykiss* and coho salmon *O. kisutch* after being decompressed from 4.0 ATA (30 m). The presence of emphysema in rockfish that were captured at depths where other organs were unaffected, suggests the heart ventricle, being a highly perfused organ, is more sensitive to excessive gas exposure than other less well-perfused organs (Speare 1998). As rockfish undergo forced decompression, the excess gas from the swimbladder enters into the body cavity through

leakage or rupture (Hannah et al. 2008b). This would expose the internal organs to high gas pressures. Yellowtail rockfish likely did not have emphysema in the heart because gas pressures in the body cavity did not build up to the same levels as in the other species. It is unknown how emphysema in the heart ventricle tissue will affect fish performance in the field. Emboli in the rete mirabile and vessels of the head kidney were present in all species of rockfish, possibly indicating that these organs are also sensitive to high gas pressures when rockfish are captured from depths greater than 35 m. The rete mirabile is a gas-concentrating organ that allows a fish to inflate its swimbladder. Because it is the gas in the swimbladder which expands as a fish is brought to the surface, gas pressure in the swimbladder can increase dramatically, thus it is not surprising to find emboli in the rete mirabile as gas could be forced back into solution within the rete. The presence of emboli in the rete mirabile and head kidney could block blood flow and it is also possible that emboli could coalesce to block blood flow in progressively larger vessels (Bouck 1980), if left untreated.

Little work has been done at the histological level in fish with regard to decompression, and our research is the first work to show emboli in the rete mirabile and head kidney. Emboli have been observed with histology in the heart ventricle, atrium, and bulbus areteriosus (Edsall and Smith 1991, Smith 1988), gill filaments (Edsall and Smith 1991, Smith 1988, Speare 1998), in the adipose tissue posterior to the choroid in the eye, and between the choroid and retina in the eye (Smith 1988, Speare 1998) of fish suffering

from gas bubble disease. However, with gas bubble disease, fish are exposed to supersaturating concentrations of gas originating from the water for long periods of time, and in decompression fish are exposed to acute high gas concentrations originating from their swimbladder for short periods of time. This difference in the mechanism of gas exposure likely explains why we did not observe other organs such as the gill and eye affected by decompression.

Our results suggest some species of rockfish may be more susceptible to injury from barotrauma as a result of different membrane qualities and the depth at which rockfish are neutrally buoyant when captured. In rockfish with more elastic or thin swimbladder and pharyngo-cleithral membranes, excess gas may escape more easily and result in less internal exposure to high gas pressures. In rockfish with thicker and less elastic membranes, excess gas cannot escape as easily and can cause greater injury. In addition, the importance of depth of capture as an explanatory variable for barotrauma injury is likely a result of the amount of swimbladder gas present in a fish's swimbladder at the time of capture. Because gas volume expands exponentially with decreasing pressure, a rockfish that is neutrally buoyant at a shallow depth will have a lower volume of gas at the surface than a rockfish (with a similarly sized swimbladder) that is neutrally buoyant at a deep depth (Boyle 1675). When fish are captured from greater depths, they are exposed to high internal gas pressures for longer periods of time, which likely results in the increasing number of barotrauma injuries observed with increasing depths of capture.

Because the depth of neutral buoyancy plays a role in the severity of barotrauma injury, another interesting question is what determines the depth of neutral buoyancy for a rockfish? Alexander (1966) suggests that fish which "undertake extensive vertical migrations have neutral buoyancy only at the top of their vertical range." This is because in fish with a closed swimbladder, the swimbladder sets the upper limit to how far they can ascend without losing control of their buoyancy and floating to the surface. Although fish expend more energy maintaining their position in the water column when negatively buoyant, when negatively buoyant they are free from the restriction of their swimbladder making them positively buoyant. Parker et al. (2006) points out though, that there is little evidence a rockfish has volitional control over the volume of gas in their swimbladder. They suggest that buoyancy in rockfish is more likely a slow and continuous process of gas secretion and resorption that occurs as a result of the external gas pressure. But because gas resorption rates are much faster than gas secretion rates in rockfish (McElderry 1979), rockfish that make vertical migrations are more likely to be neutrally buoyant near the top of their vertical range than at their mean depth (Parker et al. 2006), which agrees with Alexander's observations.

Recovery after Recompression

Our recompression experiments tested the ability of black rockfish to recover from simulated decompression (from 35 m) and subsequent recompression. These

experiments isolated the effect of barotrauma only, and did not take into account the additional stressors of thermal shock (as a result of the temperature differential between the depth of capture and surface), hooking, air exposure, and handling, that rockfish captured in the wild would experience. Additionally, during the recovery period, rockfish did not experience predation or the challenge of finding food. It is difficult to conduct a field study to follow the macroscopic to microscopic recovery of rockfish after recompression in the long term though, because it would be necessary to relocate, re-capture, and sample fish at depth. In laboratory settings, we can monitor rockfish recovery more easily, and know that any injuries we observe are the result of barotrauma only, and not other factors fish experience in the field.

With this in mind, our studies showed black rockfish are quite resilient and have potential for recovery in laboratory conditions when recompressed after simulated decompression. At the macroscopic level, most treatment rockfish experienced a ruptured swimbladder after decompression; this did not heal in a 20 – 50% of fish after 31 days. At the microscopic level we found few differences between treatment and control fish, although there were differences between experimental fish and field baseline fish, indicating handling stress likely played a greater role than barotrauma. At the molecular level, we found several genes from the innate immune system up-regulated in treatment fish after barotrauma, however these genes returned to neutral regulation in most fish after the 31 day recovery period.

At the macroscopic level, rockfish exhibited no external signs of barotrauma when recompressed shortly after forced decompression. This finding has also been documented by several other studies (Parker et al. 2006; Hannah et al. 2007; Jarvis et al. 2008). However internally, most black rockfish decompressed from 35 m did have a ruptured swimbladder, which was slow to heal. By the end of the 31 day recovery period, 20 – 50% of rockfish still had a ruptured swimbladder and the outer layer of the swimbladder, the tunica externa, had not healed in 80% of fish. Similar experiments on black rockfish decompressed from 4.0 ATA (30 m) also showed that 23% of rockfish still had ruptures in their swimbladder after a 21-day recovery period (Parker et al. 2006). Although a ruptured swimbladder is not lethal, rockfish with a ruptured swimbladder will not be able to maintain neutral buoyancy because they will not be able to hold gas in their swimbladder. This may lead to a compromised ability to find prey and escape predation because of a greater expenditure of energy will be needed to maintain a position in the water column. This may affect semi-pelagic species to a greater degree than demersal species because the latter do not make as frequent of forays into the water column, but tend to stay close to the bottom. Semi-pelagic fish who attempt to maintain their habits of schooling, diving and hunting above the seafloor may succumb to exhaustion. In addition, a ruptured swimbladder may lead to an increased opportunity for pathogen infection (Kent et al. 2005) and could affect the ability of rockfish to communicate. Rockfish use their swimbladder for sound production, often in reponse to agnostic

encounters and for territorial defense (Hallacher 1974, Sirovic and Demer 2009). In other fish species, sounds are also produced during feeding and mating (Myrberg and Lugli 2006). Thus, a ruptured swimbladder could affect a rockfish's ability to interact socially. This would be a good area for future study. We do not know how a ruptured swimbladder may translate to mortality rates in the wild, but likely it will be dependent on the conditions at the time of release. A good sign of recovery at the macroscopic level was the resumption of feeding in the majority of rockfish. Some rockfish were also observed challenging other rockfish for food, which suggests they would be recovered enough to capture live prey as well. Cessation of feeding is a common response to stress, and the resumption of feeding is often used an an indicator of recovery.

At the microscopic level, the only injuries associated with barotrauma were in the rete mirabile. Two rockfish had severe injury to the rete mirabile, which included tearing of the tissue and hemorrhaging. No rockfish in the recompression experiments had emboli in the rete mirabile though, such as we observed in some rockfish that were sampled immediately after decompression. In addition, no rockfish in the recompression experiments had emboli in the head kidney or emphysema in the heart ventricle either, which we observed in several rockfish that were sampled immediately after decompression. This suggests that minor injuries caused by emboli or emphysema may heal quickly in rockfish once they are recompressed. For example, it is known that zebrafish *Danio rerio* can regenerate injured cardiac muscle (Lepilina et al. 2006, Poss et

al. 2002, Raya et al. 2003) and the same may be true for rockfish. We also did not find any evidence of injury due to barotrauma in the liver, eye, or gonad, which supports our earlier finding from the decompression studies.

Blood chemistry in rockfish also showed no differences between treatment and control fish, although there were some differences between field captured fish and rockfish used in our experiments. Plasma enzymes were highly variable, even among field baseline rockfish, thus it is possible a larger sample size is needed to detect differences among treatments using plasma enzymes, or that the two tissues where the highest concentration of of these particular enzymes are (liver and heart), were uninjured. Metabolites and cortisol showed no difference between treatment and control fish, but did show differences between fish used in experiments and field baseline fish from the field. This suggests the handling and feeding stress experienced by rockfish in the experiments affected metabolite and cortisol levels more than barotrauma. We were unable to feed rockfish while they were in the pressure chambers, thus both treatment and control rockfish experienced a two-week fasting period. In addition, experimental rockfish experienced netting, brief air exposure, and transport to and from the pressure chambers. There was also no difference in IGF-1 between treatment, control and field baseline fish in the recompression experiments, suggesting growth rates were also not affected by acute barotrauma.

At the molecular level, we identified six genes associated with the innate immune system that were up-regulated in the liver due to barotrauma. This is a significant finding because we were unable to detect differences between treatment and control fish using blood plasma samples. All six genes were significantly elevated in treatment rockfish compared to control rockfish at day 3 post-barotrauma, even after accounting for the handling stress both treatment and control fish experienced during the experiments. These genes were no longer elevated by days 15 and 31 post-barotrauma, indicating the immune system returned to control levels in treatment rockfish over time. In addition, up-regulation of complement C1q was related to the presence of a ruptured swimbladder in rockfish. This provides further support that these genes are responsive to barotrauma injury in rockfish.

In conclusion, recompressed rockfish will not be as competent as uncaptured rockfish because of unhealed swimbladders, possible injury to the rete mirabile in some fish, and up-regulation of the innate immune system. However even rockfish that are injured will have a chance at survival they would not otherwise have if they were not recompressed. The lack of mortality during the experimental period, the resumption of feeding, the ability of most swimbladders to hold gas again, the lack of a difference in blood chemistry measures between treatment and control fish, and the return of immune genes to neutral regulation are all good indicators that black rockfish are quite resilient and have the potential to recover from barotrauma.

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