

**Oxidative Stress in Juvenile Salmon in Response to Elevated
Oxygen Levels in the Stockton Deep Water Ship Channel
created by the Discharge of Hyperoxic Waters From a
Demonstration Aeration Facility**

Submitted to:



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ACRONYMS

AB	Assay Buffer
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ANOVA	Analysis of Variance
BML	Bodega Marine Laboratory
BHT	Butylated hydroxytoluene
CABA	Center for Aquatic Biology and Aquaculture
CAT	Catalase
CO ₂	Carbon Dioxide
CP	Carbonyl-modified Protein
ddH ₂ O	Distilled, Deionized Water
DNPH	Dinitrophenyl hydrazine
DO	Dissolved Oxygen
DPS	Distinct Population Segment
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DWR	Department of Water Resources
DWSC	Deep Water Ship Channel
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
ESA	Endangered Species Act
ESU	Evolutionary Significant Unit
Expt	Experiment
ft	foot, feet
GPx	Glutathione Peroxide
GR	Glutathione Reductase
GSH	Glutathione, reduced
GSHt	Glutathione, total
GSSG	Glutathione, oxidized
GSTNB	mixed disulfide of TNB (5-thio-2-nitrobenzoic acid)
HB	Homogenizing Buffer
HCl	Hydrochloric Acid
HRP	Horseradish Peroxidase
H ₂ O ₂	Hydrogen peroxide
Lab	Laboratory

K-PO ₄	Potassium Phosphate Buffer (a combination of mono- and di-potassium phosphates to attain desired pH)
m	meter(s)
M	Molar (moles/liter)
MDA	Malondialdehyde
mg	milligram
mg/L	milligrams per liter
min	minute(s)
μl	microliter
ml	milliliter
μM	micromolar
mM	millimolar
MPA	meta-Phosphoric acid
mU	milliunits (of enzyme activity)
nm	nanometer
nmol	nanomoles
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
NADP ⁺	Nicotinamide Adenine Dinucleotide Phosphate, oxidized
Na-PO ₄	Sodium Phosphate Buffer (a combination of mono- and di-sodium phosphates to attain desired pH)
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
N ₂	Nitrogen
O ₂	Oxygen
O ₂ ⁻	Superoxide anion
PBS	Phosphate Buffered Saline
pH	Power of Hydrogen
PSU	Practical Salinity Unit (seawater ~33 psu)
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SSA	Sulfosalicylic Acid
TAOC	Total AntiOxidant Capacity
TBARS	ThioBarbituric Acid Reactive Substances
TCA	Trichloroacetic acid
TEA	Triethanolamine
TNB	5-thio-2-nitrobenzoic acid

U	Units (of enzyme activity)
USFWS	U. S. Fish and Wildlife Service
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
XO	Xanthine Oxidase

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Oxidative Stress in Juvenile Salmon in Response to Elevated Oxygen Levels in the Stockton Deep Water Ship Channel created by the Discharge of Hyperoxic Waters From a Demonstration Aeration Facility

EXECUTIVE SUMMARY

This study was commissioned by the California Department of Water Resources (DWR) to determine whether a Demonstration Aeration Facility U-tube oxygenation system that was designed to elevate dissolved oxygen (DO) in the Stockton Deep Water Ship Channel (DWSC) of the San Joaquin River would cause oxidative or other damage to outward migrating juvenile salmon. We conducted two separate studies, a laboratory study to define specific effects of hyperoxia on juvenile salmon in the absence of other stressors and a second study that used the knowledge gained from the laboratory study to determine whether the U-tube oxygenation system adversely impacted juvenile salmon. This report presents data that show hyperoxia up to 400% DO (≈ 40 mg/L) for 5 days did not cause juvenile Chinook salmon mortality nor did it hinder ability of fish to withstand transfer to full strength seawater. It was determined that fish responded to hyperoxia by activating protective antioxidant mechanisms, but there was no oxidative damage to tissues or organs (Table 1). Finally, results of an *in situ* hyperoxic exposure in the DWSC confirmed these results and indicated that fish were no less able to tolerate multiple stressors under hyperoxic conditions in the DWSC than they were under ambient conditions.

Table 1 is a summary of oxidative stress assays comparing results of adaptive assays with damage assays for fish from the laboratory experiments and the field study. (+) is an assay result that indicates an increased response in hyperoxic fish while (–) denotes no difference between hyperoxic and control fish. TAOC = Total Antioxidant Capacity; GLUT RATIO = Glutathione, Reduced:Oxidized Ratio; ENZYMES = Catalase, Superoxide Dismutase, and Glutathione Peroxidase activity; TBARS = Thiobarbituric Acid Reactive Substances.

Table 1. Summary of Results

	ADAPTIVE RESPONSES				INDICATORS OF DAMAGE			
	TAOC	GLUT RATIO	ENZYMES		CARBONYL PROTEIN	TBARS	HISTOLOGY	SALINITY CHALLENGE
Lab	+	+	–		–	–	–	–
Field	–	+	–		–	–	–	–

DO in the DWSC historically dropped to 5 mg/L or less between May and August and to 6 mg/L between September and November. This seasonal hypoxia is caused by continuing urban and agricultural water and land use throughout the San Joaquin River drainage; thus, low DO is primarily anthropogenically derived. When the Environmental Protection Agency (EPA) mandated that DO levels below 5 mg/L had to be mitigated since they were detrimental to freshwater organisms, DO in this portion of the San Joaquin River had to be addressed. Of particular concern was the fact that two of the

organisms that utilize the San Joaquin River are salmonids. Both reproductive adult and out-migrating juvenile Central Valley Fall-run Chinook salmon (*Oncorhynchus tshawytscha*) and Central Valley Steelhead (*Oncorhynchus mykiss irideus*) use the San Joaquin River as part of their migratory route to and from upstream spawning areas in the San Joaquin River Basin tributaries. The National Marine Fisheries Service (NMFS) has identified the Central Valley Fall-run Chinook salmon evolutionarily significant unit (ESU) as a species of concern, while the Central Valley steelhead distinct population segment (DPS) is listed as threatened under the Endangered Species Act (ESA). In addition, critical habitat for the Central Valley steelhead DPS was designated in the Delta. Therefore, water quality is important to the well-being of threatened Central Valley steelhead as the region of concern serves both as a migratory corridor and a rearing habitat for fish transiting the area. Low DO in the DWSC has specific application to salmon. Juveniles avoid low DO and thus may be prevented from passing through the DWSC when hypoxic conditions predominate. Conversely, if avoidance does not occur, growth is hindered at 5 mg/L and mortality can occur beginning at 4 mg/L. To counteract seasonal hypoxia DWR designed and built the Demonstration Aeration Facility at the Stockton DWSC; operational plans call for it to be activated when DO in the DWSC approaches 5.0 mg/L. The aerator releases supersaturated DO water along a 200 ft diffuser pipe point source at about 35-40 mg/L of O₂ and elevates the DO at decreasing concentrations across the DWSC; e.g. at 10-20 ft from the diffuser end, DO is increased by 4-5 mg/L thereby alleviating hypoxic conditions. However, near the diffuser origin where supersaturated water is being released, DO in the DWSC could potentially be elevated to *hyperoxic* levels (DO elevated over normoxic conditions) creating a different suite of physiologically stressful conditions.

Two general effects of hyperoxia have been reported. One is gas bubble disease, though this is caused by and requires supersaturation of both dissolved oxygen and dissolved nitrogen, not just DO. Supersaturation of O₂ and N₂ leads to formation of gas bubbles in the head and peritoneal lining of the body cavity and oral cavity. This can impair gas exchange across the gill epithelium, disrupt swimming behavior, and generally disturb physiological homeostasis. The second potential effect of hyperoxia is oxidative stress. When pro-oxidants such as reactive oxygen species (ROS; e.g. superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite) exceed the body's ability to counteract ROS (antioxidant capacity), cell damage can occur that ultimately can lead to disruption of overall homeostasis. Many types of stress can result in oxidative stress; for example, starvation, toxic chemicals, temperature stress, osmoregulatory stress, even mental stress from crowded or confined conditions can lead to oxidative stress.

The salmon that were used in both laboratory and field studies were obtained from the U.S. Fish and Wildlife Service's (USFWS) Coleman National Fish Hatchery on Battle Creek in Anderson, CA. Hyperoxic freshwater was generated at the University of California's Bodega Marine Laboratory (BML) for the laboratory studies in a custom-built oxygenation system that used Mazzei inline venturi injectors to deliver medical grade O₂ (the highest purity available) to multiple tanks. Target DO levels in laboratory studies were 200% and 400% of ambient normoxic levels and exposure times were 1, 2 and 5 days. For the field study, fish were acclimated to DWSC water for 4 days, placed into cages and submerged in the DWSC with the DWR Demonstration Aeration Facility operating for 5 days. To analyze effects in both studies we employed a hierarchal

approach. First, we assayed for activation of antioxidant activity defense mechanisms that prevent cellular damage. These included analyses of total antioxidant capacity (TAOC), antioxidant enzyme activities (superoxide dismutase, SOD, catalase, Cat, glutathione peroxidase, GPx) and glutathione ratio (reduced GSH:oxidized GSSG). Secondly, fish were assayed for cell and tissue damage using biochemical assays (carbonyl proteins, CP and lipid peroxidation as TBARS) and histological methods, as well as organism-level stress upon saltwater challenge.

Under controlled laboratory conditions in which elevated DO was the only stressor manipulated, survival and ability to withstand the additional physiological stress of a saltwater challenge were not impacted. The suite of oxidative stress assays used in the laboratory study did reveal an increase in general antioxidant activity (plasma TAOC and kidney glutathione ratio), but no oxidative stress damage in any of the tissues assayed: gill, liver, kidney, or skeletal muscle. The adaptive increase of plasma TAOC (antioxidant compounds and/or enzymes) with increasing DO levels is a positive response to oxidative challenge. The drop in kidney glutathione ratios with increasing DO indicates a response to increased oxidative stress and is not deleterious in and of itself. In fact, the response to increased oxidative stress stopped there. Neither histological analysis nor the oxidative damage assays, carbonyl protein and TBARS, showed any significant difference between hyperoxia and normoxia. Damage to gills and other organs would have been evident if hyperoxia had caused gas bubble disease; and gill damage, whether evident or not in histological preparations, would have been reflected in an inability to withstand the saltwater challenge.

The field study in the Stockton DWSC, in contrast to the laboratory experiments, tested juvenile Chinook salmon ability to tolerate elevated DO under prevailing river conditions, i.e. in the presence of other oxidative stressors. Fish were placed into cages that were situated both over the U-tube oxygenator outlet diffuser and control sites a mile upstream and downstream from the diffuser. Ambient river water DO concentrations during the field study ranged from 7-8.5 mg/L at the control sites to a high of 13.8 mg/L at the test site adjacent to the U-tube oxygenator outlet diffuser. Thus, hyperoxia in the field study was less than 200% of ambient. At the end of the 5-day experiment, no fish carcasses were observed in the cages; however, none of the cages contained the initial stocking number of fish. The initial stocking density was 30 fish per cage. Due to safety concerns for personnel, cages were not monitored during the experiment, thus it was not possible to determine whether initial miscounts were made during initial stocking of the cages, predation occurred within the cages that reduced initial stocking numbers, fish had escaped from the cages, and/or mortality had occurred due to water conditions (high DO or other stressors). For these reasons our conservative approach treated missing fish as mortality of unknown cause. Compared to control site cages, there were more missing fish from experimental diffuser cages. However, no correlation with the orientation of the cage within a rack, nor with the distance of the rack to the diffuser existed. The 150 ft rack possessed the highest mortality of the experimental racks, yet it recorded the lowest average DO, similar to upstream and downstream control levels. There were documented mortalities during the post-hyperoxic saltwater challenge, however, there were no significant differences between experimental and control fish. Overall, 75% of fish survived the saltwater challenge and 2/3 of the mortalities were ascribed to trauma from a

dorsal fin clip during pre-saltwater challenge marking of outside cage fish from all locations.

The tissues and assays chosen to evaluate oxidative stress in the field study were based on laboratory results. These were plasma TAOC, liver carbonyl protein and TBARS, liver and kidney glutathione ratio, and an assay not performed on laboratory samples, blood glutathione ratios. Oxidative stress assays indicated that fish were experiencing oxidative stress during the field study; however, there was no correlation with proximity to the Demonstration Aeration Facility's U-tube oxygen diffuser, the source of hyperoxic DO. Blood glutathione levels were significantly lower in fish from all river stations compared to those from fish that remained at BML as non-DWSC controls but not different from each other. Kidney glutathione ratios were lower in some experimental cages than others but were just as low at the control sites! Thus fish that experienced 5 days caged in the DWSC were clearly exposed to oxidative stress, but unlike in the laboratory experiments this exposure was not correlated with hyperoxia. Additionally, as in the laboratory experiments, carbonyl protein and TBARS levels and histological analysis indicated that there was no cell or tissue damage.

In conclusion, there was no evidence of additive or synergistic effects of hyperoxia created by the Demonstration Aeration Facility's U-tube oxygen diffuser with other oxidative stresses which could have adverse effects on migrating salmon.

INTRODUCTION

The San Joaquin River and its tributaries have in recent history supported four anadromous salmonid species, Spring-run, Fall-run, and late Fall-run Chinook salmon (*Oncorhynchus tshawytscha*) and Steelhead (*Oncorhynchus mykiss irideus*). One of these, *Oncorhynchus mykiss irideus*, is currently listed as threatened by NOAA's National Marine Fisheries Service (NMFS) under the Endangered Species Act. The Sacramento – San Joaquin Delta and portions of the San Joaquin River basin were designated as critical habitat for this Central Valley steelhead DPS (Distinct Population Segment). Spring-run Chinook salmon were extirpated from the San Joaquin River watershed in the late 1940's and the remaining two runs of Chinook salmon, the Fall-run and late Fall-run exist in substantially reduced numbers from historic populations in today's environment. Due to construction of impassable dams in the late nineteenth and early twentieth centuries on the basin tributaries flowing through the low foothills of the Sierra Nevada mountain range on the eastern edge of the San Joaquin Valley, access to historic spawning and rearing habitats in the higher elevations of this mountain range was compromised. With the advent of large water storage and diversion projects in the upper San Joaquin River watershed in the mid-twentieth century, the main stem of the San Joaquin River from the confluence of the Merced River upstream to approximately Mendota Pool has been consistently dewatered, preventing upstream or downstream migration of salmonids to the spawning reaches immediately below Friant Dam. The upstream reach of the river from Friant Dam to near Mendota Pool has ceased to serve as spawning and nursery grounds for anadromous salmonids, including Fall-run and Spring-run Chinook salmon and Central Valley steelhead. The valley floor segment of the San Joaquin River from the southern Delta to the confluence of the Merced River near Hills Ferry is now the only migratory route for reproductive adults moving upstream to spawning grounds in the tributaries (*i.e.*, Stanislaus, Tuolumne, and Merced rivers) as well as the Merced River Hatchery, and for juveniles on their seaward migration from those tributaries and hatchery.

Due to a plethora of compounding factors, including dredging and channelization, unscreened water diversions, and agricultural and urban land use, water quality in this system has continued to worsen, now to the extent that the river's role as a migratory corridor for adult and juvenile salmonids is dangerously compromised. One measure of water quality that directly affects the success of these species is dissolved oxygen (DO). The Environmental Protection Agency (EPA) has mandated that DO levels below 5 mg/L are detrimental to freshwater organisms and must be addressed. Federal and State agencies monitor DO at various locations in the San Joaquin River system. The Federal and State agencies have used this data to identify that DO levels of 5mg/L or less routinely occur between May and November beginning at the south end of the Stockton Deep Water Ship Channel (DWSC) and extending at least 14 miles downstream (California Department of Water Resources, 2010; also see Lee and Jones-Lee 2004; Volkmar and Dahlgren, 2006; Jones & Stokes, 2007). These decreased dissolved oxygen levels (hypoxia) can overlap with the seaward migration of salmonid juveniles (Whitmore, et al., 1960; Gowdy and Grober, 2003; Jassby and Van Nieuwenhuyse, 2005).

Hypoxia in salmonid streams can occur naturally on a seasonal and annual basis, and it seems likely that juveniles have evolved behavioral and physiological mechanisms to deal with low DO. Minimum DO requirements vary with the salmonid species and are related to water temperature; however, there is a general consensus that growth is retarded when DO is below 5 mg/L and mortality ensues somewhere between 2-4 mg/L (Davison, et al., 1959; Whitmore, et al., 1960; Herman, et al., 1962). Pre-smolt salmonids avoid low DO where possible, but if unable to avoid areas of low DO, they will accelerate smoltification and migrate rapidly through hypoxic regions (Whitmore, et al., 1960; Gowdy and Grober, 2003; Jassby and Van Nieuwenhuyse, 2005). If smolts experience prolonged exposure to hypoxic conditions, hypercapnia (increased carbon dioxide concentration in the blood stream) can ensue. If prolonged, this can lead to acid-base imbalance, reduced respiratory activity, and ultimately decreased growth and/or mortality (Cruz-Neto and Steffensen, 1997; Breitburg, 2002). Both avoidance and mortality have been reported as effects of DWSC hypoxia on migrating pre-smolt salmon (Whitmore, et al., 1960).

The California Department of Water Resources (DWR) constructed a Demonstration Aeration Facility that contains a U-tube oxygenation system designed to elevate DO in the DWSC during periods of unacceptable hypoxia. This is defined by the San Joaquin River Basin Plan as less than 5.0 mg/L DO (see Lee and Jones-Lee, 2003; Jones & Stokes, 2007) and the objective of the plan is to insure that DO is at 5.0 mg/L or above from December through August and at 6.0mg/L for the rest of the year. The aerator discharges water supersaturated with dissolved oxygen at a concentration of 35-40 mg/L along a 200 ft long diffuser pipe and elevates DO concentrations across the DWSC through mixing and diffusion. The aerator design criteria targets raising the channel DO levels 4 to 5 mg/L (ICF International 2010) at a distance of 10-20 ft from the diffuser.

Hyperoxia is rare in natural freshwater systems, but does occur in conjunction with air supersaturation created by power generation turbines at dams and has also been reported due to oxygenation of either fish culture tanks or transport water. Two effects of hyperoxia on fish have been suggested. One is gas bubble disease, a direct effect most often seen in fish exposed to gas supersaturated water that not only contains high DO, but also elevated partial pressures of nitrogen gas, N₂. Pathologies of gas bubble disease include gas bubbles in the body, head and peritoneal lining of the body cavity, and oral cavity (Elton, et al., 1997; Domitrovic, et al., 2000). Gas bubble disease impairs gas exchange across the gill epithelium, can disrupt swimming behavior, and generally disturbs physiological homeostasis. The second potential effect of hyperoxia is oxidative stress, which is induced by an increase in pro-oxidants or reactive oxygen species (ROS, e.g. superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite) within cells and tissues (Droge, 2001). The generation of certain ROS occurs during normal cell respiration and metabolism and some free radicals are actually essential to many normal biological processes including secondary messenger roles within cells and immune functions (Rice-Evans, et al., 1991). However, ROS can alter the structure and integrity of biological molecules leading to loss of cell integrity and cell death. To counteract this, cells utilize antioxidants, like ascorbic acid, α -tocopherol, and glutathione to scavenge and eliminate ROS. When an imbalance between ROS and protective removal mechanisms is created, cell and tissue damage can occur. Assays to detect oxidative stress can be divided into two categories, those that measure antioxidant

activity and thus show protective responses, (Das and Fanburg, 1992; Jornot and Junod, 1992), and those that measure cell, tissue, or organ damage from oxidative stress (Buss, et al., 2000; Rice-Evans, et al., 1991).

All types of antioxidant mechanisms and levels of pro-oxidant damage have been demonstrated in teleost fish (Martínez-Álvarez, et al., 2005). Studies on hyperoxia-induced oxidative stress in salmonid pre-smolts have reported both no effect and general physiological effects that have varied with age and species. One hundred seventy-five percent DO under culture or farming conditions resulted in 3% mortality, along with reduced growth in surviving Atlantic salmon parr (Espmark and Baeverfjord, 2008). In another study, there were no mortalities of Atlantic salmon pre-smolts exposed to up to 180% DO for 22 days and then subjected to a seawater challenge (Friddel, et al., 2007). This same study did show susceptibility of hyperoxic fish to IPN virus inoculation, however, the authors concluded that this could only be attributed to increased stress which included CO₂ and general metabolite build-up in the experimental tanks. Brauner (1999) reported mortality in coho salmon (*Oncorhynchus kisutch*) smolts after seawater transfer, but also showed mortality in normoxic control fish. Growth during hyperoxia has been investigated in a number of studies and most of these have reported either no effect or reduced growth, regardless of species (Beschta, et al., 1987; Brauner 1999; Olsvik, et al., 2005; Espmark and Baeverfjord, 2008). In one study Atlantic salmon exhibited increased growth rates over 30 days of hyperoxia (123% DO; Hosfeld, et al., 2008).

Hypercapnia resulting from hyperoxic conditions has been implicated as the source of stress in fish exposed to hyperoxia. Hypercapnia is reported to occur from either of two sources during hyperoxic exposure. One is reduced ventilation in fish that leads to a build-up of blood CO₂ resulting in a decrease of blood pH; this has been hypothesized to interfere with osmoregulation and ability to withstand osmotic stress (Heisler, 1993; Brauner, et al., 2000; Hosfield et al, 2008). This internal source of hypercapnia, however, is compensated for within 24 hrs (Brauner, et al., 2000; Hosfield, et al., 2008). The second source is a CO₂ increase in fish culture water tanks from an increased metabolic rate. Atlantic salmon were unable to tolerate high salinity after culture in hyperoxic and hypercapnic water, however, osmoregulatory ability was not affected by hyperoxia if normal CO₂ levels were maintained (Hosfeld, et al., 2008).

This project was undertaken to determine effects of the DWR Demonstration Aeration Facility U-tube oxygenation system on Fall-run Chinook salmon, *Oncorhynchus tshawytscha*, in the DWSC and to determine if any observed physiological responses were directly or indirectly caused by hyperoxia, and not by other oxidative stressors. We conducted two separate studies. The first study was a laboratory study to enumerate specific effects of hyperoxia on juvenile salmonid fish in the absence of other oxidative stressors. The second study used the knowledge gained from the laboratory study to define whether or not the U-tube oxygenation system in the Stockton DWSC adversely impacted juvenile salmonids. We employed a hierarchical approach to analyze the effects on fish that were exposed to hyperoxia that began with survival. If fish survived, we assayed them for biochemical and physiological responses. As Table 2 describes, first we looked for activation of antioxidant activity defense mechanisms that prevent cellular damage. Second, fish were assayed for cell and tissue damage using biochemical and histological methods. Third, we subjected fish to osmotic stress by subjecting them to a

saltwater challenge. Assessment of hyperoxic impact included analyses of total antioxidant capacity, antioxidant enzyme activities (superoxide dismutase, catalase,

Table 2. Description of oxidative stress assays performed on juvenile Chinook salmon.

1. Antioxidant Defenses	
a. Total Antioxidant Capacity, TAOC, in Plasma	The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system. These include antioxidant enzymes, macromolecules such as albumin, ferritin, etc., an array of small molecules such as ascorbic acid, α -tocopherol, β -carotene and reduced glutathione.
b. Superoxide Dismutase, SOD	SOD is the major intracellular antioxidant enzyme in aerobic cells. It reduces superoxide radicals to hydrogen peroxide.
c. Glutathione Peroxidase, GPx d. Glutathione, reduced, GSH Glutathione, oxidized, GSSG	Glutathione peroxidase acts to remove hydrogen peroxide and lipid hydroperoxides (a product of lipid peroxidation that causes additional oxidative damage) by utilizing reducing equivalents supplied by GSH. Oxidized glutathione, GSSG, is converted back to GSH by the NADPH-dependent enzyme, glutathione reductase, maintaining the levels of reduced glutathione for continued antioxidant defense.
e. Catalase, CAT	Catalase catalyzes the conversion of hydrogen peroxide to water. The normally low concentration of hydrogen peroxide is reduced in cells by GPx; however, if the concentration is raised, e.g. by oxidative stress, then catalase becomes important.
2. Oxidative Damage	
a. Lipid Peroxidation, TBARS	Lipid peroxide decomposition yields Thiobarbituric Acid Reactive Substances, TBARS, such as malondialdehyde and lipid hydroperoxide which react colorimetrically with thiobarbituric acid and so can be quantified using this assay.
b. Protein Carbonyl Content	Oxidation transforms certain side-chain amine groups on several amino acids into carbonyls. Protein carbonyls react with dinitrophenyl hydrazine, DNPH, and the resulting protein-hydrazone can be analyzed spectrophotometrically at 360-385nm using this assay.

glutathione peroxidase), direct oxidative damage (protein carbonyls and lipid peroxidation), and organismal-level impacts (histological damage and ability to withstand a saltwater challenge).

This report describes the results of these studies that tested whether hyperoxia under controlled laboratory conditions and hyperoxia created by the Demonstration Aeration Facility in the DWSC produces oxidative stress in Chinook salmon juveniles. The report details results that show fish responded to extreme hyperoxia under laboratory conditions by activating protective antioxidant mechanisms, but also shows that there was no oxidative damage to tissues or organs. Finally, results of an *in situ* hyperoxic exposure in the Stockton DWSC confirm these results and suggest that fish are no less able to tolerate multiple stressors under hyperoxic conditions than they are in normoxic water.

MATERIALS AND METHODS

LABORATORY STUDIES

Four laboratory experiments were conducted with juvenile Chinook salmon. The first three were designed as preliminary experiments to test the oxygenation system, refine oxidative stress assays, and to determine effects of different hyperoxic exposure times on fish survival and response to oxidative stress. The fourth experiment (***Complete Hyperoxic Experiment***) utilized the knowledge gained from the first three experiments regarding methodology and assay protocols. It: 1) examined effects of hyperoxia on survival; 2) determined ability of juvenile fish to withstand a post-hyperoxic saltwater challenge; and 3) determined if adaptive response or oxidative damage had occurred as a result of hyperoxia.

Oxygenation system. The oxygenation system was a flow-through system that consisted of nine 72-liter covered treatment tanks plumbed in groups of three. One group received ambient DO freshwater (~8 mg/L) defined as 100% Control DO, and the two other groups received increasing concentrations of hyperoxic DO water, one targeted at 200% of ambient DO and one at 400% of ambient DO. The oxygenation system used Mazzei® inline venturi injectors that delivered medical grade O₂ to incoming freshwater that was directed to one of two holding reservoirs after oxygenation, one for 200% and one for 400% ambient DO (Fig. 1). These in turn supplied treatment tanks that were plumbed in groups of three such that each DO concentration treatment could be conducted in triplicate. Water DO and temperature were recorded daily in each treatment tank during laboratory experiments using Midge oxygen probe systems (Eureka Environmental Engineering) that provided continuous data in 15 minute intervals and using Polaris hand-held probes (OxyGuard, Inc.) which were used for discrete daily readings. Each probe was calibrated according to the manufacturer's instructions and tested in the oxygenation system. Accuracy of the Polaris probes was confirmed with the Winkler test.

Acquisition and Acclimation of Salmon. Juvenile Fall-run Chinook salmon were obtained from the U.S. Fish and Wildlife Service's (USFWS) Coleman Fish Hatchery in Anderson, CA, and transported by truck in freshwater to the Bodega Marine Laboratory (BML). Fish were held in tanks supplied with flow-through freshwater under the care of Kristin Arkush and Anne McBride (UC Davis Animal Protocol #12840). Fish were apportioned to holding tanks in the BML Salmon Containment Facility and acclimated to on-site freshwater and held for up to 14 weeks for the laboratory experiments. Temperature and make-up water rate were controlled and monitored via WonderWare & Siemens PLC computer software. Dissolved oxygen levels were monitored four times daily using a Polaris oxygen probe. Fish were fed BioDiet starter from BioOregon at 2-4% of body weight with automatic feeders. Weight and fork length in a subset of fish were recorded on arrival (n=72) and at approximately monthly intervals during acclimation and holding (n=30-39).

Laboratory Preliminary Experiment 1: Oxygenation System and Assay Test. Six fish were incubated in freshwater at each of the three DO levels (100, 200, 400%) for 5 days. Polaris probes were used to record DO and temperature twice daily. Tanks were examined daily for mortalities and after 5 days the fish tissues were collected for refinement of oxidative stress assays.

Laboratory Preliminary Experiment 2: Saltwater Challenge. Two treatments were used, ambient and 400% DO for 5 days. Polaris probes were used to record DO and temperature twice daily. Sample size was 6 fish in each treatment tank. The tip of the caudal fin was clipped in the ambient fish and all fish were transferred to a single tank of freshwater for the saltwater challenge. Salinity was increased at 2-3 psu (practical salinity unit) per hour over the first 24 hours after which fish were held at 33 psu for at least 24 hours.

Laboratory Preliminary Experiment 3: Hyperoxic Duration Test. A full complement of triplicate treatments in each of three DO levels (100%, 200%, 400%) was used. Polaris probes were used to record DO and temperature twice daily and Midge probes provided continuous readings in the elevated DO tanks. Sample size was 30 fish in each treatment tank. At 1, 2 and 5 days ten fish were removed from one replicate tank for each of the 3 DO levels and used for oxidative stress assays. Another 10 fish were removed for histological analysis. The remaining 10 fish from each DO level were fin-clipped for identification and transferred to a single tank of full-strength seawater (100% DO) for a 24 hr, 33 psu saltwater challenge.

Laboratory Experiment 4: Complete Hyperoxic Experiment. Thirty fish in triplicate (90 fish per DO concentration) were transferred to the treatment tanks containing 100%, 200%, or 400% DO water and maintained for 5 days. Daily records were kept on water temperature, water DO, and fish mortality. After 5 days, 10 fish from each of nine tanks (30 fish per DO treatment) were removed and used for oxidative stress assays. The freshwater in each tank was lowered to ~20 liters and replaced with seawater at 2 liters per minute and the 20 remaining fish in each tank were subjected to the saltwater challenge.

FIELD STUDY

Field Study Site. The field study was conducted in the Stockton DWSC, upstream, downstream, and adjacent to the Port of Stockton's West Complex Docks 19/20 on Rough and Ready Island. The downstream end of the West Complex dock is the site of the DWR Demonstration Aeration Facility U-tube oxygen diffuser. The diffuser is mounted beneath the West Complex at Docks 19/20 (10ft below the surface at low tide) and is 200 feet long, 30 inches in diameter, and contains eighty, 6-inch ports spaced at 2.5 foot intervals and alternately directed horizontally and at a 45° angle toward the bottom of the channel (ICF International, 2010).

Cage Design and Deployment. The field study was conducted using Fisherman's Circular Live Bait/Fish Holding Pen Cages (dimensions 2 ft tall x 2 ft diameter, 1cm

mesh size, item # 2424, Catch-n-Bait Supply Co, Punta Gorda, FL. 34601) to hold juvenile fish. Triplicate cages were rafted together and buoyed to float them at approximately 1 meter below the surface regardless of tidal height (Fig. 2 and Appendix I). Cage racks were deployed in the Stockton DWSC at five sites (Fig. 3, 4, 5 and Appendix I). Control cage racks were anchored 1 mile upstream and 1 mile downstream from the beginning of the oxygen diffuser. Experimental cages were deployed above the diffuser pipe at 50 ft, 100 ft, and 150 ft from the beginning of the diffuser. Cages in each experimental rack were spaced such that the middle cage was over the diffuser, the outside cage was 1.5 m from the diffuser toward the channel, and inside one cage was 1 m from the diffuser toward the shore. Midge remote DO sensors were placed in one cage of each cage rack such that continuous 15-minute interval DO and temperature data was recorded at both control sites and all three distances along the diffuser pipe.

Fish Acquisition, Transport and Acclimation. A second group of juvenile Chinook salmon was transported from the USFWS Coleman National Fish Hatchery and maintained as previously described in freshwater at BML for 30 days. Approximately 600 fish were transported to the Port of Stockton in aerated and chilled (11°C) freshwater. Upon arrival in Stockton, fish were transferred to a circular 500 gallon tank with re-circulating (2 gal/hour), aerated San Joaquin River water that was taken from the West Complex Dock 19/20. Fish were acclimated to San Joaquin River water for 4 days. During acclimation the following were documented daily: time, water flow (yes/no), aeration (yes/no), water clarity, water temperature, DO (mg/L), the number of moribund fish observed, and the number of fish mortalities observed.

River Exposure. After four days of acclimation to San Joaquin River water, the DWR diffuser was activated and fish were transferred to previously deployed cages in the Stockton DWSC. Thirty fish were transported to each of the 15 cages in 5 gal lidded buckets containing river water by boat. Fish were transferred by slowly pouring the fish from buckets into the cages through hatched cage openings after which hatches were closed and secured. The study, exposure of caged fish to diffuser discharge, continued for 5 days (approximately 10 tidal oscillations through the experimental area). This approximated the expected duration of time that juvenile salmon would be expected to be in the proximity of diffuser during their downstream migration (Jeff Stuart, pers. comm.). Due to possible danger under the dock while the U-tube oxygenator was in operation, cage rafts were not monitored during the 5-day experiment.

On day 5 of the field study, all remaining surviving fish were netted from the cages, one cage rack at a time, into lidded buckets and returned to the dock. Ten fish per cage were sacrificed and tissues sampled for use in histology and oxidative stress assays (see ***Collection, Preparation, and Handling of Tissues for Assays*** below). The remaining fish from each cage (20 or less) were fin clipped to identify which cage they were from, grouped by cage rack, and subjected to a 24 hr saltwater challenge. The saltwater challenge in Stockton was achieved by immersing fish into a tank that contained synthetic seawater derived from mixing Instant Ocean™ with ambient San Joaquin River water to a salinity of 24 psu. The tank was aerated and DO and temperature were monitored continuously with a Midge remote sensor. At the termination

of the 24 hr saltwater challenge mortalities and moribounds were scored and living fish were euthanized with MS222.

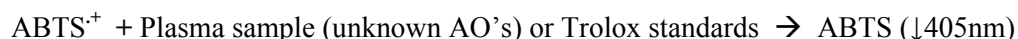
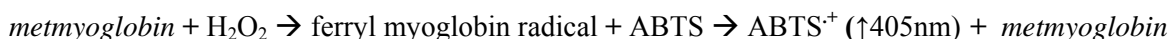
HISTOLOGY

Tissue for histological examination from both the laboratory and field studies was removed from fish as pictured in Appendix II. Samples of the gill, kidney, liver and muscle were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C, then rinsed in PBS, placed in tissue cassettes, and sent to Diagnostic Pathology Medical Group, Inc. (Sacramento, CA) in ethanol for preparation of slides for histological analysis. Tissue was sectioned at 5 microns and stained with hematoxylin and eosin.

OXIDATIVE STRESS ASSAYS

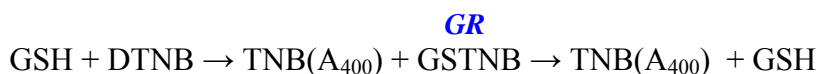
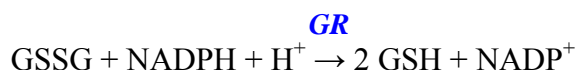
Collection, Preparation, and Handling of Tissues for Assays. Fish were sacrificed by overdose in MS222 and weight and fork length recorded. The tail fin was amputated and blood was collected in a heparinized capillary tube and then transferred to a microfuge tube. In both the laboratory and field experiments, an aliquot of blood was spun at 1000 x gravity (1000xg) for 10 minutes (at 4°C in the lab, ambient temperature in the field) and the clear plasma layer removed to a labeled tube, flash frozen in liquid nitrogen and stored at -80°C for determining plasma total antioxidant capacity. In the field, a 10 microliter (μ l) aliquot of whole blood was placed in a 0.6 milliliter (ml) microfuge tube containing 1 μ l glutathione scavenging reagent (1-methyl-2-vinyl-pyridium trifluoromethane sulfonate, M2VP, Oxford Biochemicals, kit GT30) and vortexed to mix thoroughly. A 5 μ l aliquot of whole blood was placed in a tube without scavenging reagent and both tubes were flash frozen in liquid nitrogen and stored at -80°C for determining blood glutathione ratios. Gill, liver, kidney and skeletal muscle were dissected out (Appendix II), rinsed in PBS, blotted, placed in a labeled microfuge tube, flash frozen in liquid nitrogen, and stored at -80°C for use in the remaining oxidative stress assays (see *Tissue preparation for individual assays* below).

Plasma Total Antioxidant Capacity (TAOC). The total antioxidant capacity considers the cumulative effect of all antioxidants present in plasma and so is an excellent measure of relevant physiological response to oxidative stress. Total antioxidant capacity of the plasma was measured in salmon from laboratory preliminary experiments 1 and 3, the complete laboratory experiment 4, and the field study with the ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) decolorization assay of Rice-Evans and Miller (1994) using [Cayman Chemical Kit #709001](#). In this assay metmyoglobin reacts with hydrogen peroxide, H₂O₂, to form the ferryl myoglobin radical which converts ABTS to the radical cation ABTS^{•+}. ABTS^{•+} is monitored at a wavelength of 405 nanometers (nm) in a plate reader. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble derivative of Vitamin E and is used to create a standard curve of “antioxidant capacity,” represented by the amount of ABTS^{•+} converted back to ABTS in a given amount of time. Plasma total antioxidant capacity is expressed as Trolox-equivalents. The overall reaction is as follows:



A standard curve from 0.0 to 0.33 millimolar (mM) Trolox was prepared from a 1.5mM stock solution in assay buffer consisting of 5mM potassium PO₄, pH 7.4, 0.9% NaCl, 0.1% glucose. A 96-well plate was loaded with duplicate 10 μ l aliquots of standards and samples (diluted 1:1 with assay buffer), 10 μ l metmyoglobin and 150 μ l ABTS (as provided in kit) and the reaction started with the addition of 40 μ l 441micromolar (μ M) H₂O₂. The plate was mixed and incubated for 5-10 minutes and the absorbance read at 405nm on a Bio-Rad Model 550 plate reader (Hercules, CA). Absorbance was plotted versus Trolox concentration and the regression line used to calculate Trolox-equivalents in Chinook salmon plasma.

Blood Glutathione Ratio (Reduced GSH:Oxidized GSSG): Glutathione, GSH, is an important antioxidant. Often present in mM concentrations, it is a major redox buffer in cells (reviewed by Arrigo, 1999). GSH, alone or as a cofactor for GPx enzymes, reduces ROS and products of oxidative stress such as lipid hydroperoxides. In the process, GSH is oxidized (oxidized glutathione - GSSG), and is returned to its reduced form by glutathione reductase (GR), utilizing NADPH as a cofactor. Total and oxidized glutathione was measured in the blood of juvenile salmon from the field study using the enzymatic recycling method of Tietze (1969) with updates on the method from Griffith (1980), Anderson (1985) and Baker, et al., (1990) and [Oxford Biomedical Research kit # GT30](#) as follows:



The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) to produce the yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB, that is concomitantly produced, is reduced by glutathione reductase (GR) to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 400nm provides an accurate estimation of GSH in the sample. Measuring initial concentrations of GSSG is accomplished by immediately derivatizing the GSH in the sample with M2VP (1-methyl-2-vinylpyridinium trifluoromethanesulfonate), a novel thiol-scavenging reagent from Oxford Biomedical Research, to prevent its subsequent oxidation to GSSG. The same enzymatic recycling method can then be used to measure the much lower concentrations of GSSG.

Blood samples derivatized in the field for GSSG measurement (10 μ l blood + 1 μ l M2VP) were removed from the ultracold freezer and an additional 1 μ l of M2VP was added followed by thawing, mixing and incubating at room temperature for 2 minutes.

29 μ l of cold 5% MPA (meta-phosphoric acid) was added and the tube vortexed for 15 seconds and then centrifuged at 1000xg for 10 minutes. A 10 μ l MPA extract was diluted with 90 μ l assay buffer (100mM sodium phosphate, 1mM EDTA, pH 7.5) for a final pH between 6.5-7.0 and 40x dilution. Blood samples for the measurement of GSH (5 μ l, no M2VP) were thawed, mixed, combined with 15 μ l cold 5% MPA, vortexed and centrifuged at 1000xg for 10 minutes. 5 μ l MPA extract was added to 620 μ l assay buffer for a final 500x dilution.

Reduced (GSH) and oxidized (GSSG) glutathione standards were prepared to mimic samples. For GSH standards, 1mM GSH in blood-equivalent buffer (BEB: 50mM potassium phosphate, 0.5mM EDTA, pH 7.0) was diluted to 10 μ M GSH with assay buffer. Plain assay buffer was used as blank solution since MPA was diluted to a negligible 0.03% in blood samples. For GSSG standards, 5 μ l M2VP was added to 25 μ l of 200 μ M GSSG in BEB then combined with 70 μ l 5% MPA and 900 μ l assay buffer for 5 μ M GSSG final (40x dilution). GSSG blank solution consisted of 100 μ l BEB + 10 μ l M2VP + 290 μ l 5% MPA + 3.6ml assay buffer. Standard curves were produced by combining standard and blank solutions to achieve a range of GSSG concentrations from 0.0 to 1.5 μ M and GSH concentrations from 0.0 to 3.0 μ M.

The assay was run twice, first to measure total glutathione, reduced plus oxidized (GSHt) and then to measure GSSG alone. For both GSHt and GSSG assays, 25 μ l blank, standard or sample were pipetted in duplicate into wells of a 96-well plate. A 25 μ l aliquot of DTNB (in Na₂PO₄ with EDTA and ethanol from Oxford Biomedical Kit #GT30) and a 25 μ l aliquot of glutathione reductase (in Na₂PO₄ with EDTA from Oxford Biomedical Kit #GT30) were added to each well and the plate was incubated for 2 minutes at ambient room temperature. A 25 μ l aliquot of NADPH (1.33mg/ml) was added and the absorbance at 400nm recorded for 5 minutes in a Tecan GENios Microplate Reader using MagellanTM Data Analysis software.

Calculating the GSH and GSSG concentrations and the GSH/GSSG ratio requires four steps: 1) determining the reaction rate, 2) constructing calibration curves, 3) calculating GSHt and GSSG concentrations, and 4) calculating the GSH/GSSG ratio. The reaction rate was determined by plotting absorbance at 400nm versus time for each GSH and GSSG concentration and running a linear regression for each concentration to generate the linear equation that describes the Abs vs Time relationship, where $\text{abs}(y) = \text{rate}(m) * \text{min}(x) + b(\text{y-intercept})$. The slope (m) of this line is the reaction rate. The slope was either provided by the Magellan program or determined using SigmaPlot software for graphing and linear regression. The reaction rates were then plotted versus their GSHt or GSSG concentration. Another regression was run where $y = \text{reaction rate}$ and $x = \text{standard concentration}$. The resulting standard curve ($y = mx + b$) was then used to calculate glutathione concentration in the sample wells. The reaction rate (abs vs. time) was determined for each sample well as described above and the rate was then fed into the standard curve as y and the equation solved for x , glutathione concentration. Duplicates were averaged and values multiplied by the appropriate dilution factor to give μ M GSHt or GSSG in the blood. Reduced glutathione is equal to total glutathione (GSHt) minus twice oxidized glutathione (2*GSSG) because every GSSG molecule contributed 2 GSH molecules to the total glutathione concentration. The glutathione ratio, reduced to oxidized, was determined with the following equation:

$$([\text{GSHt}]-2*[\text{GSSG}])/[\text{GSSG}]$$

Tissue Preparation for Individual Assays. Tissues were prepared for oxidative stress assays according to Luschak, et al. (2005). Samples were removed from the ultracold freezer and allowed to thaw on ice. One ~100 milligram (mg) portion of each sample was homogenized in homogenization buffer (HB: 50mM K·PO₄, 0.5mM EDTA, pH 7.0) plus protease inhibitor cocktail (Sigma #P2714) at a concentration of 100mg wet weight tissue/ml HB using Kontes Duall 1ml glass tissue grinders. Two 250μl aliquots of homogenate were combined with 250μl 20% trichloroacetic acid (TCA) in labeled 1.5ml microfuge tubes, vortexed, placed on ice for 10 minutes, then centrifuged at 10,000xg for 5 minutes. The pellets were assayed for carbonyl proteins the same or next day and the supernates for thiobarbituric acid reactive substances (TBARS), the same day as homogenization. The remaining homogenate was centrifuged at 10,000xg for 10 minutes at 4°C. The supernate was divided into 4 aliquots with these minimum volumes for each assay: 10μl for catalase (Cat), 100μl for superoxide dismutase (SOD), 50μl for glutathione peroxidase (GPx), and the aliquots flash-frozen in liquid nitrogen before storage at -80°C.

A second portion of tissue, minimum 30mg, was homogenized in ice-cold 5% 5-sulfosalicylic acid (SSA) at a concentration of 100mg/ml, placed on ice for 15 minutes and centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant fraction was transferred to a new tube, flash-frozen and stored at -80°C for the glutathione ratio assay.

Carbonyl Protein including Total Protein. Carbonyl-modified proteins (CP) were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) following [protocol #10005020](#) from Cayman Chemical Co. CPs were measured in gill, liver, kidney, and muscle from the complete laboratory experiment 4 and in liver from the field study. One of the two identical tubes of TCA-precipitated protein was derivatized with DNPH while the second tube was the non-derivatized control. Both samples were resuspended in 0.5ml 2.5 normal (N) hydrochloric acid (HCl) in Kontes glass tissue grinders, transferred to clean labeled microfuge tubes and combined with either 0.5ml 2mM DNPH (derivatized, “S”) or 0.5ml 2.5N HCl (control, “C”). After incubation for 1 hour in the dark with rotation, samples were centrifuged at 13,000xg for 5 minutes at ambient room temperature, the supernates carefully discarded and the pellets resuspended in 1ml of a 1:1 mix of absolute ethanol and ethyl acetate. After washing 5 minutes with rotation, samples were re-centrifuged, supernates discarded and pellets resuspended in the ethanol:ethyl acetate solution. This wash step was repeated two times, carefully removing the ethanol:ethyl acetate supernate each time in order to completely remove unreacted DPNH.

The final pellets were resuspended in 6 molar (M) guanidine-HCl (guan-HCl) in 20mM Na·PO₄, pH6.5, with thorough vortexing and incubation for 10 minutes with rotation at ambient room temperature before centrifuging for 10 minutes at 10,000xg to remove any leftover debris. A 220μl aliquot from each sample and control tube was pipetted in duplicate into a 96-well plate and the absorbance of the resulting 2,4-dinitrophenylhydrazones were measured at 370nm in the Tecan GENios Microplate Reader using MagellanTM Data Analysis software. The amount of CP in the resulting supernatants was calculated using a molar extinction coefficient of 22×10³M⁻¹ cm⁻¹

(Lenz, et al., 1989) adjusted to $0.011\mu\text{M}^{-1}$ for the path length of the solution in the well. The average value of “C” wells were subtracted from the average value of “S” wells and divided by the adjusted extinction coefficient to yield carbonyl concentration (nmol CP/ml guan-HCl supernate).

Protein concentration was determined in the non-derivatized samples by pipetting $50\mu\text{l}$ from each control well into duplicate wells of a UV-transparent 96-well plate, adding $150\mu\text{l}$ 6M guanidine-HCl and measuring absorbance at 280nm in the Tecan plate reader. A standard curve was generated with bovine albumin in 6M guanidine-HCl and the protein concentration calculated. Carbonyl concentration was then divided by protein concentration to yield carbonyl content (nmol CP / mg protein). Total (TCA-precipitated) protein was expressed as mg protein/mg wet weight tissue.

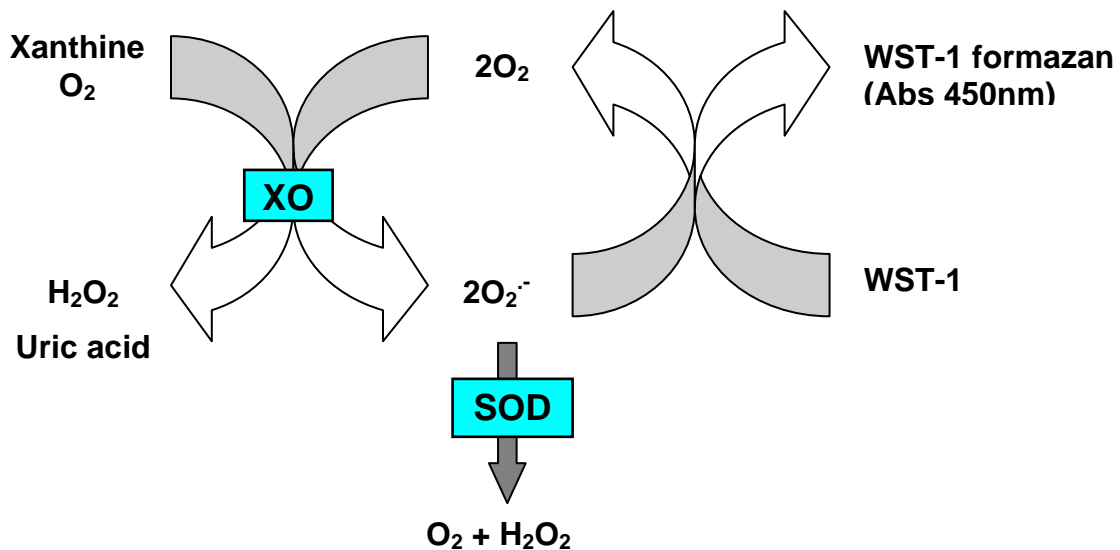
Lipid Peroxidation, TBARS (ThioBarbituric Acid Reactive Substances). The decomposition of lipid hydroperoxides produces low molecular weight products, including malondialdehyde, which can be measured by the TBARS assay (Rice-Evans, et al., 1991). We used [Cayman Chemical Kit #10009055](#) but followed the [Animal Models of Diabetic Complications Consortium \(AMDCC\)](#) protocol. TBARS was measured in gill, liver, kidney, and muscle from complete laboratory experiment 4 and in liver from the field study. Duplicate $200\mu\text{l}$ aliquots of the sample TCA supernates and malondialdehyde (MDA) standards ($0\text{--}100\mu\text{M}$ in 10% TCA) were combined with an equal volume of 0.53% thiobarbituric acid in 0.1N HCl containing 0.01% BHT to prevent in-tube peroxidation during heating and then heated for 1 hour at 100°C in a hot block. After cooling, $150\mu\text{l}$ duplicate aliquots were pipetted into wells of 96-well plate and absorbance measured at either 540nm in the Bio-Rad plate reader or at 560nm in the Tecan plate reader. MDA concentration was determined using the standard curve and lipid peroxidation expressed as nanomoles MDA per mg wet weight tissue.

Tissue Glutathione Ratio (Reduced GSH:Oxidized GSSG). Although both GSH and GSSG occur in tissues, GSH is by far the predominant form (Anderson, 1985). Greater than 99.5% of tissue total glutathione, GSht, (i.e., GSH + GSSG, in GSH equivalents) is in the form of GSH. Since GSH readily oxidizes nonenzymatically and especially rapidly at $\text{pH} > 7$ to GSSG, and because it is a good substrate of γ -glutamyl transpeptidase (as is also GSSG), biological samples must be acidified quickly to reduce oxidation of GSH to GSSG and also to inactivate γ -glutamyl transpeptidase (Anderson, 1985).

Glutathione was measured in SSA extracts from gill, liver, kidney, and muscle from laboratory experiment 1 and in liver and kidney from the field study. Kidneys are especially high in γ -glutamyl transpeptidase which will cleave GSH as frozen tissue is warmed. To prevent this, kidneys were removed from the ultracold and placed directly in a container of liquid nitrogen. One sample at a time was removed, quickly weighed and homogenized in 5% SSA as it thawed. SSA extracts were derivatized with either 2-vinyl pyridine (lab expt 1) or M2VP (field study) while simultaneously increasing pH to ~ 6.5 with triethanolamine (TEA diluted 4x in distilled, deionized water, ddH₂O) and then diluting 5-8x with assay buffer (see blood glutathione assay) to measure GSSG concentration. To measure total GSH concentration non-derivatized SSA extracts were diluted 100x in assay buffer.

Total (GSht) and oxidized glutathione (GSSG) were measured using the same assay as for blood glutathione with the following differences: 50 μ l of a solution of 0.6mM DTNB and 4U/ml GR was added to wells followed by 25 μ l of sample, standard or blank with 2 minutes shaking at ambient room temperature. The reaction was initiated with 25 μ l of 1.8mM NADPH for a final concentration of 0.6mM per well. The reaction was monitored, graphed, and analyzed as described for blood glutathione. Standards for the GSSG assay were prepared by combining 10 μ M GSH and 40 μ M GSSG in 5% SSA in proportions to generate an initial standard curve containing 1mM GSH and from 0.0 to 36 μ M GSSG and then adding M2VP and TEA and assay buffer to more closely mimic sample extracts for a final standard curve with 125 μ M GSH and from 0.0 to 4.5 μ M GSSG. A standard curve for the GSht assay was prepared from 5 μ M GSSG stock in assay buffer diluted to 0.0 to 5 μ M GSSG (= 0.0-10 μ M GSHeq).

Superoxide dismutase (SOD). Superoxide dismutase (SOD) activity was assayed in homogenate extracts from laboratory experiments 1 and 4 using [Dojindo SOD Assay kit-WST \(#S311-08\)](#). Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion ($O_2^{\cdot-}$) into H_2O_2 and O_2 . Dojindo's kit uses a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye that absorbs at 450nm upon reduction with a superoxide anion ($O_2^{\cdot-}$). The superoxide anion is produced by the activity of xanthine oxidase (XO). SOD competes with WST-1 for $O_2^{\cdot-}$ so that the SOD activity can be quantified as an inhibition activity by measuring the decrease in the color development at 450 nm. The reaction can be depicted as follows:



Sample wells contained diluted homogenate extract (gill = no dilution, muscle = no dilution, kidney = 2x, liver = 10x), WST solution and xanthine/XO solution. Blank 1 wells contained buffer instead of sample; Blank 2 contained sample but no xanthine/XO solution; Blank 3 contained only WST and buffer. Standard wells contained SOD in a range from 0 to 100 U/ml. The plate was incubated at 37°C for 20 minutes and then

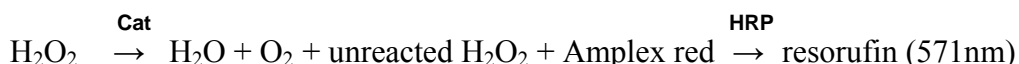
absorbance measured in the Tecan plate reader. SOD activity was calculated as % inhibition using the following equation:

SOD activity (inhibition rate %) =

$$\{[(\text{Abs}_{\text{blank 1}} - \text{Abs}_{\text{blank 3}}) - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank 2}})] / (\text{Abs}_{\text{blank 1}} - \text{Abs}_{\text{blank 3}})\} \times 100$$

A standard inhibition curve was always plotted to confirm assay performance.

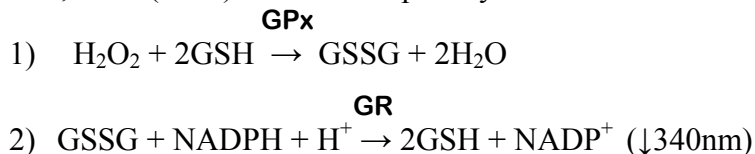
Catalase (Cat). Catalase activity was measured in homogenate extracts from laboratory experiments 1 and 4 using [Molecular Probes Amplex Red Kit](#). The following equation illustrates the chemistry of the assay:



Catalase reacts with hydrogen peroxide to produce water and oxygen and then the Amplex red reagent reacts with any unreacted H_2O_2 in the presence of horseradish peroxidase (HRP) to produce the resorufin which can be monitored at 571nm. Therefore as catalase activity increases, the signal from resorufin decreases.

Samples were diluted in 0.1M Tris-HCl, pH 7.5 (gill = 250x, liver = 1000x, kidney = 500x, muscle = 50x) and 25 μl pipetted into duplicate wells. Standards ranged from 0.0 to 750mU/ml final concentration in wells. 25 μl of 40 μM H_2O_2 was added and the plate incubated at ambient room temperature with gentle shaking. 50 μl Amplex red/HRP solution was added to each well (100 μM Amplex red and 0.4U/ml HRP final concentration) and incubated for 30 minutes at 37°C with gentle shaking. Absorbance was read at 560nm in the Tecan plate reader and catalase concentration calculated from the standard curve (ΔAbs vs. [cat]) and expressed as Units/ml.

Glutathione peroxidase (GPx). Homogenate extracts from laboratory experiment 1 were removed from the ultracold and allowed to thaw on ice. Glutathione peroxidase (GPx) activity was measured by a coupled assay with glutathione reductase (GR)-catalyzed oxidation of NADPH according to Lushchak, *et al.* (2001) with input from Flohe and Gunzler (1984), [Sigma procedure #CGP1](#), [Cayman protocol #703102](#), and Rice-Evans, *et al.* (1991). The two-step assay is illustrated below:



First, a 3-minute baseline consumption of NADPH was recorded at 340 nm using the Tecan plate reader in wells of a 96-well plate containing 50mM K_2PO_4 buffer (pH 7.0), 0.5mM EDTA, 0.25mM NADPH, 4mM sodium azide, 1U GR/ml, 15mM GSH, and a range from 5-20 μl homogenate extracts and 5-25 μl GPx standards (0, 6.6, 13.2, 19.7, 26.3 mU/ml final). Final volume in the wells was 190 μl and the temperature was held constant at 25°C. The reaction was initiated with the addition of 10 μl of 3.8mM H_2O_2 for a final concentration of 0.2mM per well.

The rate of consumption of NADPH is directly proportional to the GPx concentration over a range from $\sim 0.02 A_{340}/\text{min}$ up to $\sim 0.15 A_{340}$. The volume(s) of sample that fell in that range were used for the subsequent calculations. First, the change in absorbance (ΔA_{340}) per minute (reaction rate) for each sample well was determined by plotting the absorbance values as a function of time to obtain the slope (reaction rate) of the linear portion of the curve. Then the slope of the blank wells was subtracted from these values and the difference divided by the extinction coefficient for NADPH for the path length of the 96-well plate ($0.00373 \mu\text{M}^{-1}$). Finally, this value was corrected by the dilution factor ($0.19\text{ml}/x \text{ ml sample in well}$) to give GPx activity in mU/ml (one unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP^+ per minute at 25°C .). GPx activity was expressed as nmol NADPH/min/mg protein. A standard curve of reaction rate (slope= $\Delta\text{Abs}/\text{min}$) versus [GPx] was plotted and a linear regression run for quality control. The r^2 value was never significantly different than 1.000.

RESULTS

LABORATORY STUDIES

Oxygenation System. Prior to conducting experiments, the oxygenation system was tested and calibrated as shown in figure 6. Data from both probes showed that following each adjustment DO remained fairly constant and that the Midge probe readings were consistently below Polaris data. Appendices III & IV contain DO data from the third preliminary experiment and from the complete experiment. Six Midge sensors (one in each of the hyperoxic tanks) recorded DO at 15 minutes intervals while daily discrete readings were obtained from the Polaris sensors. Daily Polaris readings indicated that DO in replicate tanks was consistent and that DO was maintained within 10% of target DO (Fig. 7). Polaris data from the ambient DO tanks ranged from 7.73 ± 0.15 to 8.77 ± 0.12 mg/L, close to saturation at ambient temperature. Continuous data recorded by the Midge probes provided consistently lower DO readings than did the Polaris data; average daily DO was up to 25% less than DO obtained with the Polaris unit (Fig. 8). Individual probe differences between the Midge probes were also evident and these differences were repeated across experiments; for example Midge-40 consistently provided the lowest of the 200% DO readings (Appendix III).

Acclimation. Juvenile salmon were acclimated and maintained at BML from August 9, 2007 until used for laboratory DO experiments (between mid-October and December 13, 2007). There were neither mortalities, nor signs of disease during the acclimation period. Weight and fork length were measured at monthly intervals during the acclimation period. Upon arrival at BML average length and weight were 83 mm and 7.7 gm (Fig. 9) and 7.7% of the fish were greater than 100 mm length, with none over 120 mm; 100 mm is considered a minimum size for this hatchery stock to successfully smolt (Scott Foote pers. Comm.). By November 16 average length and weight were 122 mm and 24.3 gm, 96.4% measured more than 100 mm with 72% greater than 110 mm. During the last month of acclimation, the size range for the population expanded. For the first three months 86-96% of fish were clustered within a 40 mm range. This range expanded 60 mm by the end of the third month. Total size range on November 16 was 81-170 mm.

Hyperoxic Survival and Saltwater Challenges. All hyperoxic fish that were subjected to saltwater challenge in preliminary laboratory experiments survived either a 24 hour transition to full-strength seawater (33 psu) or a 24 hour challenge when transferred directly to full-strength seawater. In preliminary laboratory experiment 1, juvenile Chinook salmon tolerated an average DO of 34 mg/L (425% of control DO) at 15.5°C for five days. There were no mortalities. In preliminary laboratory experiment 2, fish tolerated an average DO of 51 mg/L (540% of control DO) at 15.1°C for five days with no mortalities and then also survived a gradual saltwater challenge (4-33 psu over 24 hours) for another 5 days with no mortalities (except for one control fish which was injured during handling). In the hyperoxic duration experiment, fish survived 5 days at 8 mg/L (100%), 23 mg/L (287%), and 37 mg/L (462%) at 14.4°C and in addition, survived

an abrupt transfer to full-strength seawater for 24 hours with only one mortality from the control tank.

In the complete laboratory experiment, all fish survived hyperoxia (20 and 37 mg/L, 250 and 462% of control DO, respectively) at 14.2°C for 5 days. The number of fish undergoing the saltwater shock (immediate exchange of seawater for freshwater) was doubled (n=20 per tank) and the number of mortalities was still low and not significant (Fig. 10, $P=0.675$, one-way ANOVA): survival was $95\% \pm 5$ (mean \pm s.d.) in the ambient DO (8 mg/L), $97\% \pm 5.3$ in the 20 mg/L DO, and $98.3\% \pm 2.8$ in the 37 mg/L DO treated fish.

Oxidative Stress Assays

Plasma Total Antioxidant Capacity, TAOC. The total antioxidant capacity represents the sum of endogenous and food-derived antioxidants. A two-way analysis of variance (ANOVA) revealed that fish in the hyperoxic duration study significantly increased their plasma TAOC between ambient (8 mg/L) and 37 mg O₂/L and between 1 and 2 days exposure (Fig. 11). Aposteriori testing (Tukey) indicated that at ambient DO, TAOC was higher after 5 days than 1 and 2 days ($P=0.001$ & 0.044) and that 1 and 2 days were not significantly different, $P=0.057$. There were no significant differences in TAOC over time at 23 mg/L, while at 37 mg/L TAOC was already greater after 2 and remained high at 5 days compared to 1 day ($P=0.006$ & 0.014) but did not significantly increase between 2 and 5 days ($P=.884$). After 1 day of hyperoxia, TAOC levels were already significantly higher at 37 mg/L than ambient ($P=0.041$) and remained so after 2 days ($P=0.023$). There were no significant differences between DO levels after 5 days.

Figure 12 shows that plasma TAOC levels in fish from the complete laboratory experiment were significantly elevated after 5 days at both 20 mg/L and 37 mg/L DO compared to ambient (8 mg/L) DO (one-way nested ANOVA with aposteriori Tukey testing).

Carbonyl Protein including Total Protein. Carbonyl groups can be formed by ROS attack on side chains of certain amino acids and are a marker of oxidative stress (Levine, et al., 1994). Neither CP concentrations nor total protein varied significantly between DO levels or between replicates within DO levels in any of the tissues in fish from the complete laboratory experiment (Fig 13a,b-Gill; Fig 14a,b-Liver; Fig 15a,b-Kidney; Fig 16a,b-Muscle) as determined by one-way nested ANOVAs.

Lipid Peroxidation, TBARS. Hyperoxic exposure did not measurably increase lipid peroxidation. There were no significant differences in thiobarbituric acid reactive substances between DO levels or between replicates within DO levels in any of the tissues in fish from the complete laboratory experiment (Figs 17a,b,c,d: Gill, Liver, Kidney, Muscle) as determined by one-way nested ANOVAs.

Glutathione Ratio (Reduced GSH:Oxidized GSSG). Hyperoxic exposure did not cause significant changes in glutathione ratios in gill, liver, or muscle in fish from the preliminary laboratory experiment (Figs. 18a,b,c,) as determined by one-way ANOVAs, but the glutathione ratio significantly decreased in kidney with hyperoxic exposure, i.e.

GSSG levels increased relative to GSH levels, indicative of response to oxidative stress (Fig. 18d).

Superoxide dismutase (SOD). There were no significant differences in SOD activity between DO levels or between replicates within DO levels in any of the tissues in fish from the complete laboratory experiment (Figs 19a,b,c,d: Gill, Liver, Kidney, Muscle) as determined by one-way nested ANOVAs.

Catalase (Cat). There were no significant differences in catalase activity between DO levels or between replicates within DO levels in any of the tissues in fish from the complete laboratory experiment (Figs 20a,b,c,d: Gill, Liver, Kidney, Muscle) as determined by one-way nested ANOVAs.

Glutathione peroxidase (GPx). There were no significant differences in GPx activity between DO levels or between replicates within DO levels in any of the tissues in fish from the complete laboratory experiment (Figs 21a,b,c,d: Gill, Liver, Kidney, Muscle) as determined by one-way nested ANOVAs.

FIELD STUDY

Acclimation. Juvenile salmon were transferred to Stockton and acclimated to DWSC water for 4 days in tanks provided with flow through river water and oxygenation with air stones. The juveniles used in the field study were smaller than those used for the laboratory studies; 94.6% of the fish measured were 70-90 mm fork length and averaged 6.9 g in weight (Fig. 9). No fish were over 100 mm fork length. No mortalities occurred during the four day acclimation.

DWSC Dissolved Oxygen Measurements. DO levels during operation of the oxygen diffuser were based on data collected by: 1) BML Midge probes that were placed in upstream and downstream control cages as well as in cages that were at the 50 ft, 100 ft, and 150 ft marks along the diffuser pipe (Figs. 3, 5 and Appendix V); 2) DWR probes that measured DO within the oxygenator pipes, and placed in the DWSC at different distances from the diffuser pipe (Appendix VI). Within the oxygenator pipe DO reached 46 mg/L. Near the diffuser pipe DO ranged between 8-14 mg/L (BML) and 12-18 mg/L (DWR). The Midge probes revealed an oscillating DO during the course of the five-day experiment (Fig. 22) that was highest 100 ft from the diffuser origin and lowest 1 mile upstream and downstream. At 100 ft, orientation to the diffuser, directly above (middle) or towards the channel (outside) made no difference in DO levels.

Fish Survival in the DWSC. Fish were not monitored during the 5-day DWSC field study. As such the only fish counts made were when fish were placed in the cages on day one (30 fish in each cage) and the number of fish that were in the cages when they were retrieved after five days. No fish carcasses were present; however, none of the cages contained 30 fish when they were retrieved and opened. We have taken a conservative approach and made the assumption that the reduced numbers of fish at termination were due to mortality by unknown cause and that carcasses were eaten and/or lost from the

cages (Table 3). Under this assumption, mortality was enhanced in the cages near the diffuser (data combined, diffuser cages vs. control cages, $P=0.038$, t-test); however, within the diffuser cages only (Fig. 23), a two-way ANOVA revealed no significant differences due either to distance from the diffuser (50, 100, 150 ft, $P=0.276$) or orientation to it (Inside, Middle, Outside, $P=0.561$).

Table 3. Juvenile Chinook salmon survival after 5 days in the Stockton DWSC and during the subsequent saltwater challenge.

Site	Elevated DO Exposure			Salt Challenge	
	# Fish in Cage	Mortality *	# for Assay	# Fish Challenged	# Mortalities
Upstream Control					
Inside (1)	30	2	10	18	2
Middle (2)	30	2	10	18	2
Outside (3)	30	3	10	17	14
Downstream Control					
Inside (1)	30	3	10	17	3
Middle (2)	30	2	10	18	1
Outside (3)	30	6	10	14	6
50 Foot - Diiffuser					
Inside	30	1	10	19	2
Middle	30	8	10	12	0
Outside	30	4	10	16	4
100 Foot - Diiffuser					
Inside	30	5	10	15	6
Middle	30	5	10	15	0
Outside	30	6	10	14	8
150 Foot - Diiffuser					
Inside	30	7	10	13	5
Middle	30	5	10	15	0
Outside	30	6	10	14	6

* No carcasses or moribund fish were present (in any cage) when cages were opened at retrieval from the DWSC. Conservative assumption was that 30 fish were placed in each cage at experiment start, but no confirmation was available.

Note: All fish in saltwater challenge were fin-clipped to identify orientation of cage during elevated DO. Outside cage fish had dorsal fin clipped. Salt challenge temperature was 16-23°C and DO was 5-7 mg/L O₂.

Saltwater Challenge. Fish that were not used for oxidative stress assays were incubated in 24 psu artificial seawater for 22 hr. Temperature during the saltwater challenge ranged from 16-23°C and DO was between 5 and 6 mg/L for the majority of the challenge (Appendix VI). The fish (12-19 from each cage) were marked by fin clip (Inside, dorsal part of tail; Middle, ventral side of tail; Outside, dorsal fin). Mortality based on cage assignment during DWSC hyperoxia exposure ranged from 0% to 82.3% (14/17 fish; see Table 3). Overall mortality for all cages was 25.1% (59/235 fish) and the highest mortalities overall were from the outside cages, regardless of location, that had been dorsal fin-clipped. Mortality did not correlate with cage proximity to the diffuser during the experiment, but rather to how fish were marked after retrieval from

cages and before placement in the saltwater challenge tanks. The dorsal fin clip was, unfortunately, made too close to the body on some of the fish resulting in almost complete removal of the fin. Thirty-eight of the mortalities (16.2% or 2/3 of the mortalities) were dorsal fin-clipped fish from the outside cages, including control cages as well as experimental diffuser cages.

Oxidative Stress Assays

Data from the field study was first subjected to a two-way analysis of variance comparing fish from cages over the U-tube oxygen diffuser. Distance from the diffuser (50, 100, 150 ft) was one treatment and orientation to the diffuser (middle, inside, outside) at each distance was the second treatment. Further analysis depended on the outcome of this 2-way ANOVA and is described for each assay.

Plasma Total Antioxidant Capacity. As the colored bars of figure 24 illustrate, the difference in the mean values of plasma TAOC among the three distances was not great enough to exclude the possibility that the difference was due to random sampling variability after allowing for the effects of differences in orientation. There was not a statistically significant difference ($P=0.208$). The difference in the mean values among the different orientations was also not great enough to exclude the possibility that the difference was due to random sampling variability after allowing for the effects of differences in distance. There was not a statistically significant difference ($P=0.530$). Lastly, the effect of different distances did not depend on what level of orientation was present. There was not a statistically significant interaction between distance and orientation ($P=0.273$).

As there was no overall significance to the differences between distances or orientations, no specific *a posteriori* testing among diffuser cages followed. However, an overall one-way ANOVA was run to compare fish in the cages from control sites, upstream and downstream river and laboratory (BML) to each other and the diffuser cages (Fig. 24 all bars). The differences in the plasma TAOC values among the cages were not great enough to exclude the possibility that the difference was due to random sampling variability; there was not a statistically significant difference ($P=0.376$).

Blood Glutathione Ratio. For this assay, reduced (GSH) and oxidized (GSSG) forms of glutathione were measured and the result was expressed as a ratio of reduced to oxidized (GSH:GSSG, Fig. 25). Oxidative stress can result in an increase of GSSG relative to GSH so that a *decrease* in the ratio is indicative of response to oxidative stress. According to the two-way ANOVA (colored bars), in the blood the difference in the mean values of the glutathione ratio between the different distances was not great enough to exclude the possibility that the difference was just due to random sampling variability after allowing for the effects of differences in orientation. There was not a statistically significant difference ($P=0.404$). Between orientations, the difference in the mean values was also not great enough to exclude the possibility that the difference was just due to random sampling variability after allowing for the effects of differences in distance. There was not a statistically significant difference ($P=0.440$). And, the effect of different levels of distance did not depend on orientation, i.e. no significant interaction between the distance and orientation ($P=0.565$).

However, when all of the cages were tested with a one-way ANOVA followed by aposteriori testing, each of the cages from the San Joaquin River DWSC had significantly lower glutathione ratios than the laboratory control from BML ($P < 0.05$, Fig. 25, all bars) suggesting that caged river fish were responding to a higher level of oxidative stress than laboratory-held fish.

Kidney Glutathione Ratio. The two-way ANOVA on glutathione ratios in kidney tissue from fish in the diffuser cages was significant for both distance and orientation (Fig. 26a). The difference in the mean values among the different levels of distance were greater than would be expected by chance after allowing for effects of differences in orientation ($P < 0.001$); and, the difference in the mean values among the different levels of orientation were greater than would be expected by chance after allowing for effects of differences in distance ($P = 0.009$). There was no significant interaction between distance and orientation: the effect of distance on glutathione ratios did not depend on orientation ($P = 0.279$).

Aposteriori testing in the form of multiple one-way ANOVAs plus Tukey tests was performed to isolate which group(s) differed from the others. Overall, 50 ft and 100 ft cages were significantly less than 150 ft cages. Among Inside cages, both 50 ft and 100 ft ratios were significantly lower than 150 ft. The same was true for Middle cages. There was no significant difference due to distance in the Outside cages. Although orientation was significant overall (two-way ANOVA, $P < 0.009$) and Middle cages were significantly lower than Inside cages (one-way ANOVA, $P < 0.047$), there was no significant difference due to orientation within a given distance from the diffuser: at 50 ft, $P = 0.100$; at 100 ft, $P = 0.161$; at 150 ft, $P = 0.078$ (all one-way ANOVAs).

Orientation data was therefore combined within distance and the diffuser cages were compared to control cages (there was also no differences between the replicate control cages at either upstream or downstream sites, $P = 0.815$ and 0.374 respectively). Figure 26b illustrates the results. A one-way ANOVA was highly significant ($P < 0.001$). Aposteriori testing revealed that the 100 ft and 50 ft diffuser cages had significantly lower ratios than the 150 ft cages and the BML control but that the upstream and downstream controls did as well indicating that the glutathione ratio decrease was not due to diffuser influence.

Liver Glutathione Ratio. Unlike kidney tissue, there were no significant differences in glutathione ratios in liver tissue regardless of diffuser position (Fig. 27, colored bars). The difference in the mean values between distances was not great enough to exclude the possibility that the difference was just due to random sampling variability after allowing for the effects of differences in orientation ($P = 0.523$). The difference in the mean values between orientations was not great enough to exclude the possibility that the difference was just due to random sampling variability after allowing for the effects of differences in distance ($P = 0.325$). Likewise, there was no interaction effect ($P = 0.539$). There were also no significant differences between any of the cages (Fig. 27, overall) including the laboratory control ($P = 0.621$).

Liver Carbonyl Protein Content. There was no indication of oxidative damage to proteins based on carbonyl protein content in liver tissue from fish positioned over the

diffuser in the DWSC (Fig. 28a, colored bars). The difference in the mean values from cages at different distances was not great enough to exclude the possibility that the difference was just due to random sampling variability after allowing for the effects of differences in orientation ($P=0.549$). The difference in the mean values among cages at different orientations was not great enough to exclude the possibility that the difference was just due to random sampling variability after allowing for the effects of differences in distance ($P=0.626$). There was also no significant interaction: the effect of distance did not depend on orientation ($P=0.997$). Likewise a one-way ANOVA comparing means among all of the river cages and the laboratory control was not significant (Fig 28a, all bars, $P=0.996$).

Liver Total Protein Content. Measuring carbonyl protein content yields a measure of total protein content in the tissue (Fig. 28b). For liver, again, there was no significant difference (colored bars) due to either distance from the diffuser ($P=0.777$) or orientation to at each distance ($P=0.694$) and there was no interaction between the two variables ($P=0.962$). Taken individually, there was also no difference in total protein content in fish regardless of cage location ($P=0.690$, Fig. 28b, overall).

Liver TBARS (Thiobarbituric Acid Reactive Substances). Lipid oxidation measured in amounts of thiobarbituric acid reactive substances present (Fig. 29, colored bars) was not significantly different in fish regardless of their position relative to the diffuser based on a two-way ANOVA. Neither distance from the diffuser origin ($P=0.692$) nor orientation to the pipe ($P=0.169$), nor the interaction between distance and orientation ($P=0.810$) significantly affected lipid oxidation levels. None of the cages had mean TBARS levels different from any of the others regardless of cage location ($P=0.602$, Fig. 31, overall).

HISTOLOGY

The results of the histopathological study of gill structure found no indication of any effect of hyperoxia in the laboratory or in the field. A total of 90 slides were reviewed from preliminary laboratory experiment 3, the hyperoxic duration study. Gills from the control, 200%, and 400% DO fish showed no indication of gas bubble disease, GBD (Fig. 30). There was no indication of hyperplasia or fusion of the secondary lamellae. We looked at slides of gill, kidney, skeletal muscle, and liver tissue and did not see any signs of necrosis, hydropic degeneration (cellular swelling), accumulation of cytoplasmic inclusions, or visual changes in cell and nuclear volume (Fig. 31). Only gill tissue was examined from the field study since no effect was found in any of the tissues from the laboratory. Gill structure showed no differences between control and diffuser sites in the field.

DISCUSSION

Oxidative stress occurs when pro-oxidant activity exceeds antioxidant response, which can take place if pro-oxidant levels increase and/or antioxidant activity is impaired (Blumberg, 2004). Although the utilization of O₂ during normal cell respiration produces pro-oxidants, it has been shown that elevated O₂ can increase pro-oxidant levels such that oxidative stress occurs. Oxidative stress can have three levels of effect on an organism. The first effect of the antioxidant response is to eliminate pro-oxidants and thus prevent oxidative damage. Elevation of total antioxidant capacity in blood, altered activity of antioxidant enzymes (e.g. SOD, Catalase, Glutathione peroxidase, Glutathione reductase), and a decrease in the reduced GSH:oxidized GSSG ratio indicate that pro-oxidant levels are elevated compared to antioxidant capacity. The second level of response occurs after the first has failed and cell damage occurs. Indicators include increases in protein carbonyls and lipid peroxidation. The last level involves larger scale general physiological effects such as perturbation of growth, impaired ability to handle other stresses, and death.

In this study, under controlled laboratory conditions in which elevated DO was the only stressor, the ability of juvenile Chinook salmon to survive and subsequently withstand the major physiological stress of direct transfer from freshwater to saltwater were not impacted. Previous studies have reported varying results regarding fish mortality in hyperoxia, however, experimental conditions of low or no flow have been routinely used to mimic fish culture or transport conditions. Exposure to hyperoxia in low flow has been shown to produce hypercapnia (increased blood CO₂) and resultant decreased blood pH which in turn causes reduced growth, decreased osmoregulatory ability, and death (Brauner, 1999; Brauner et al. 2000). However, other factors may have been involved in at least one study since normoxic control fish also died (Brauner, 1999).

The suite of oxidative stress assays used in the laboratory study revealed an increase in general antioxidant activity, but no oxidative stress damage. The two assays which indicated a response to oxidative stress were plasma total antioxidant capacity and kidney glutathione ratios. The adaptive increase of plasma TAOC (antioxidant compounds and/or enzymes) with increasing DO was a positive response to oxidative challenge. The drop in kidney glutathione ratios (reduced GSH: oxidized GSSG) with increasing DO was in response to increased oxidative stress and not deleterious in and of itself. In fact, the response to increased oxidative stress stopped there. Histology and the assays which would have detected damage, i.e. carbonyl protein and TBARS, showed no significant difference between hyperoxia and normoxia. Damage to gills and other organs would have been evident if hyperoxia had caused gas bubble disease (Domitrovic, et al., 2000). In addition, damage to gills, whether evident or not in histological preparations, would have been reflected in the fishes' ability to withstand the saltwater challenge.

The field study in the Stockton DWSC, in contrast to the laboratory experiments, tested juvenile Chinook salmon ability to tolerate elevated DO in the presence of a plethora of possible oxidative stress agents in addition to hyperoxia (see Lee & Jones-Lee, 2004). We adopted a conservative approach to determining field mortalities. There were no fish carcasses in the cages when they were retrieved; however, none of the cages possessed 30 fish, the presumed initial number of fish. Since the cages were not monitored it was not possible to determine whether initial miscounts were made, whether

predation occurred within the cages, whether fish escaped, and/or mortality occurred due to water conditions (high DO or other stressors). Additionally, mortalities from any cause could have been partially consumed and/or deteriorated to the point that carcass remnants fell out of or floated away from the cages. For these reasons our conservative approach treated missing fish as mortality of unknown cause. There were more missing fish from experimental diffuser cages, however, no correlation with orientation of cage within a rack nor with distance of rack to diffuser existed. The 150 ft rack possessed the highest mortality of the experimental racks (Fig. 25), yet it recorded the lowest average DO similar to upstream and downstream controls (Fig. 24). All of this suggests that proximity to the docks in general was more of a factor than proximity to the diffuser.

There were documented mortalities during the post-hyperoxic saltwater challenge, however, there were no significant differences between experimental and control fish. Overall, 75% of fish survived the saltwater challenge and 2/3 of the 25% mortalities can be ascribed to trauma from a dorsal fin clip during pre-saltwater challenge marking of outside cage fish from all locations. Water conditions during the saltwater challenge added additional stress to the fish. The water that was used to make the high saline water came directly from the DWSC and during the salt challenge DO averaged 5-7 mg/L while temperature ranged from 16-23°C. Thus fish went from potentially hyperoxic conditions in the DWSC to comparative hypoxic conditions with elevated temperatures. Lethal temperatures for juvenile Chinook salmon are reported to be 24-26°C (Marine and Cech, 2004), although specific ranges change with race and geography (Richter and Kolmes, 2005). Temperatures of 12° to 17°C are thought to inhibit gill ATPase activity and thus osmoregulation (McCullough, et al., 2001). Another likely possibility for mortality during the saltwater challenge was that none of the fish had reached 100mm in length, the minimum size for these Coleman Hatchery fish to successfully smolt (Scott Foote, pers.comm).

The tissues and assays chosen to evaluate oxidative stress created by the DWR diffuser in the DWSC, based on laboratory results, were plasma TAOC, liver carbonyl protein and total protein, liver TBARS, liver glutathione ratio, and kidney glutathione ratio, plus a promising new assay, blood glutathione ratios. Oxidative stress assays suggested that fish were experiencing oxidative stress during the field study, however, there was no correlation with proximity to the oxygen diffuser pipe, the source of hyperoxia. Blood glutathione ratios in all cages of fish that spent 5 days in the DWSC (upstream, downstream and over the diffuser) were significantly lower than fish that remained in freshwater at BML. Although kidney glutathione ratios were lower at the 50 and 100 ft diffuser sites than at 150 ft and BML control sites, ratios in the two DWSC control sites were just as low, indicating that the effect was not due to proximity to the diffuser. The remaining oxidative stress assays showed no significant difference between DWSC experimental fish, DWSC control fish, or BML control fish. .

Extreme hyperoxia for up to 5 days in the laboratory activated antioxidant mechanisms indicating increased ROS in juvenile Chinook salmon, yet even the highest DO level did not result in oxidative damage or mortality. This result expands on previous studies on rainbow trout that showed a 4-hour hyperoxic exposure induced protective antioxidant activities, but that no oxidative damage occurred unless ozone was also elevated (Ritola, et al., 1999; 2002). Chinook salmon migrate long distances and adapt to drastically different salinities at least twice in their life cycle, first as out-migrating

juveniles and second as reproductive adults. The environmental conditions and physiological challenges faced during these migrations undoubtedly increase oxidative stress. Analyses of Snake/Columbia River hatchery reared, out-migrating Chinook salmon juveniles detected differing levels of kidney and liver lipid peroxidative damage (Welker and Congleton, 2005). These results were from fish exposed to stresses in the “natural” environment and thus cause and effect could not be ascribed. The authors, however, hypothesized that this oxidative stress damage was caused by general stress, which could include that from starvation, gas supersaturation at dams, and internal physiological changes due to the parr-smolt transition. The implication is that multiple stresses can result in oxidative damage. Although the juvenile salmon for the Stockton DWSC caged fish hyperoxia experiment were exposed to multiple stressors (confined to cages, not fed, exposed to elevated temperature and river toxicants) in addition to hyperoxia, we saw no oxidative damage and no elevation in protective antioxidant activity or mortality that could be ascribed to hyperoxia.

In conclusion, there was no evidence that hyperoxic conditions created by the Demonstration Aeration Facility’s U-tube oxygen diffuser had any additive or synergistic effects that would increase oxidative stress on migrating salmon.

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FIGURES

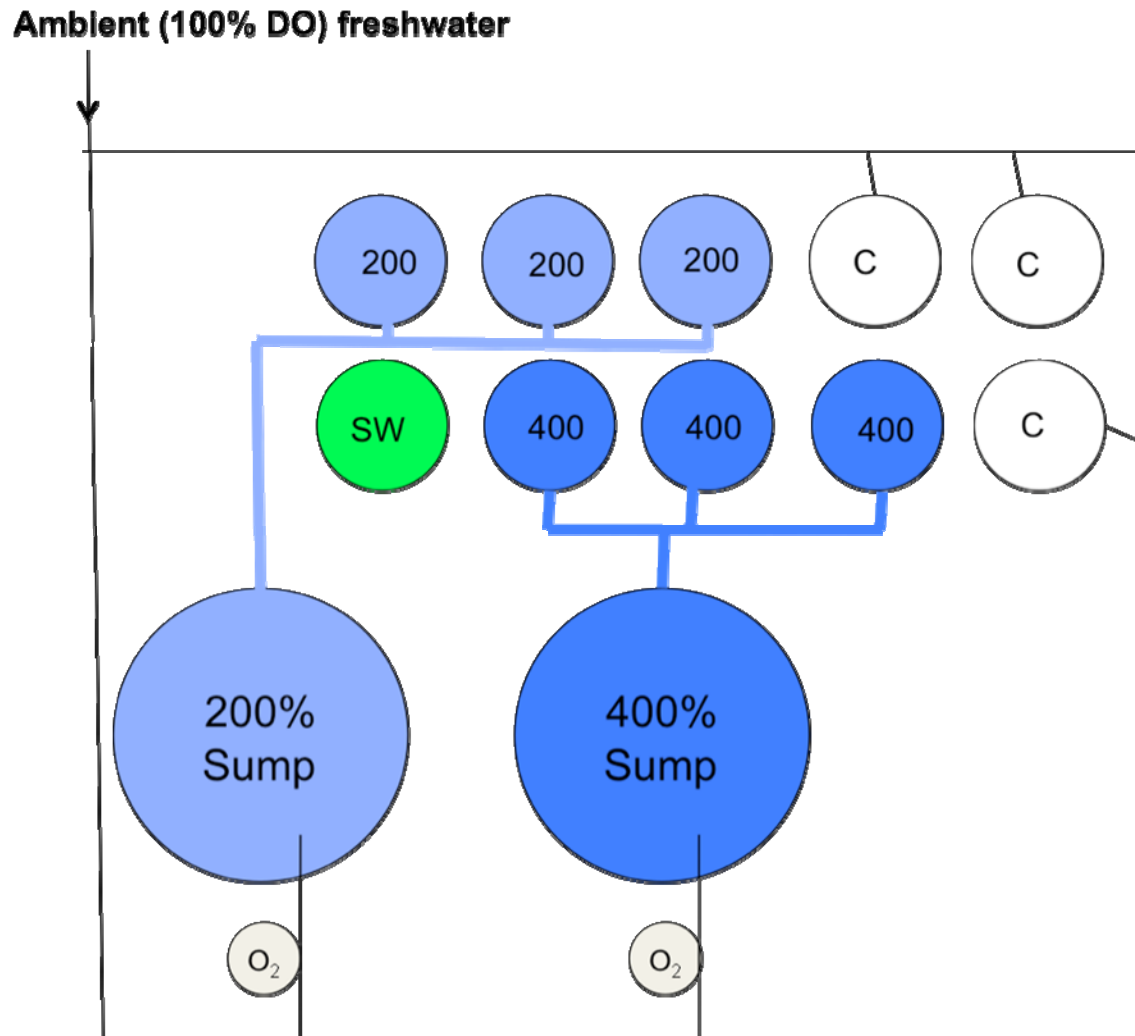


Figure 1. Layout of BML oxygenation system. Control replicate tanks (c) were supplied with ambient untreated freshwater. Hyperoxic freshwater was obtained by injecting O₂ into ambient freshwater using venturi injectors, after which the hyperoxic water was fed into a covered sump tank before being routed to nine treatment tanks.

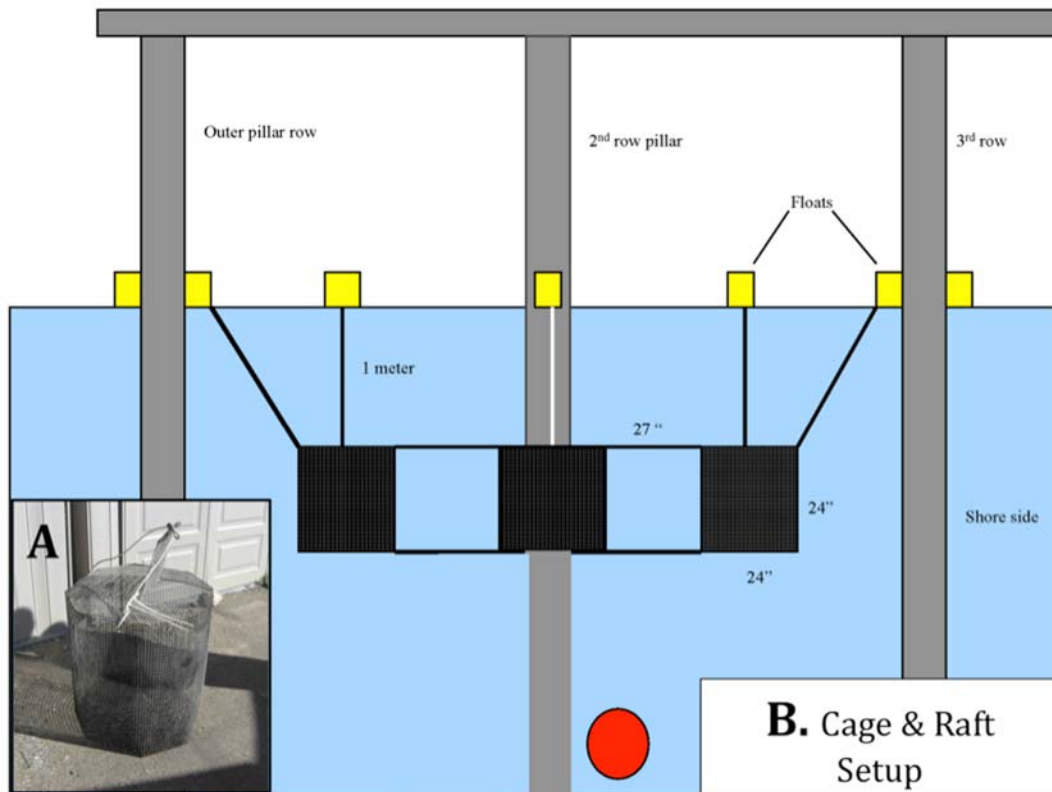


Figure 2. Salmon cage, cage/raft design, and under-dock placement at oxygen diffuser. A. Salmon cage. B. Raft design and placement. Yellow squares are floats. End floats were secured to piers such that raft could move up and down with tidal height. Red circle is the diffuser pipe.

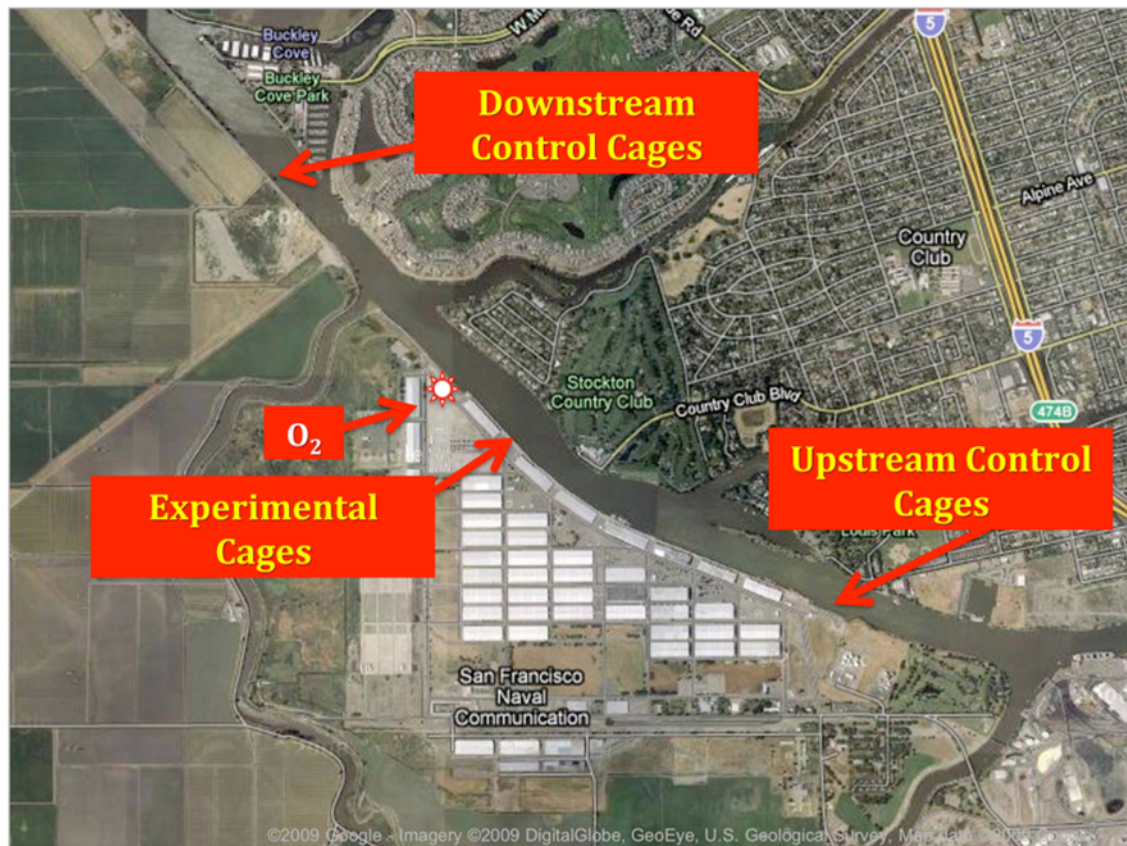


Figure 3. Position of experimental and control cage rafts in the Stockton DWSC. Control rafts were approximately 1 mile upstream and downstream from the experimental cages.



Figure 4. Position of experimental cage rafts with respect to U-tube diffuser (Red O₂).

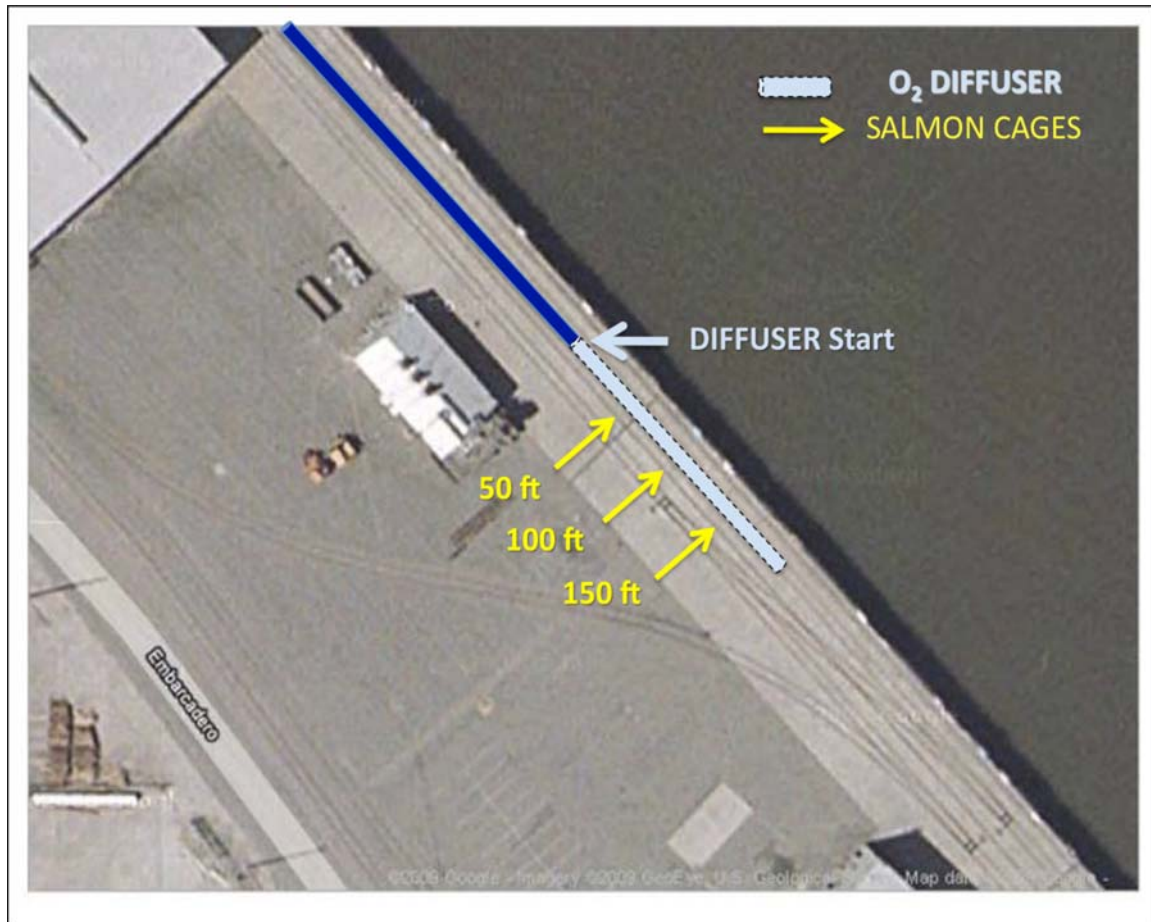


Figure 5. Three cage rafts were placed at 50, 100, and 150 feet from the beginning of the 200 foot diffuser pipe.

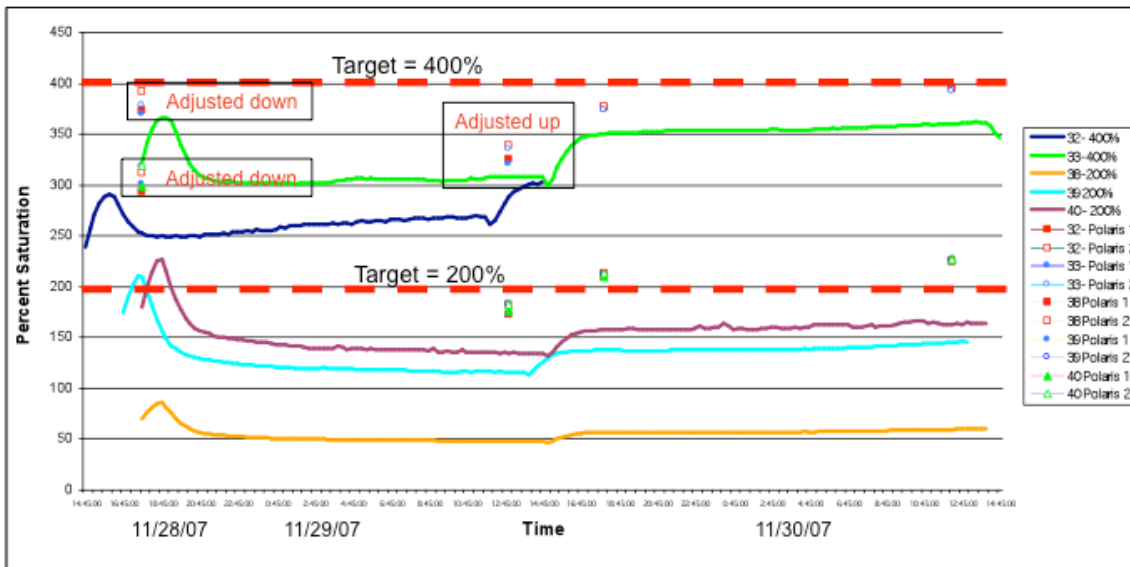


Figure 6. System and oxygen probe test. Comparative DO data from Midge and Polaris DO probes during oxygenation system testing. Adjusted up and down refers to changes made in DO of freshwater supplying tanks 38, 39, & 40 (200%) and tanks 32 & 33 (400%). Midge readings were consistently below those of Polaris units.

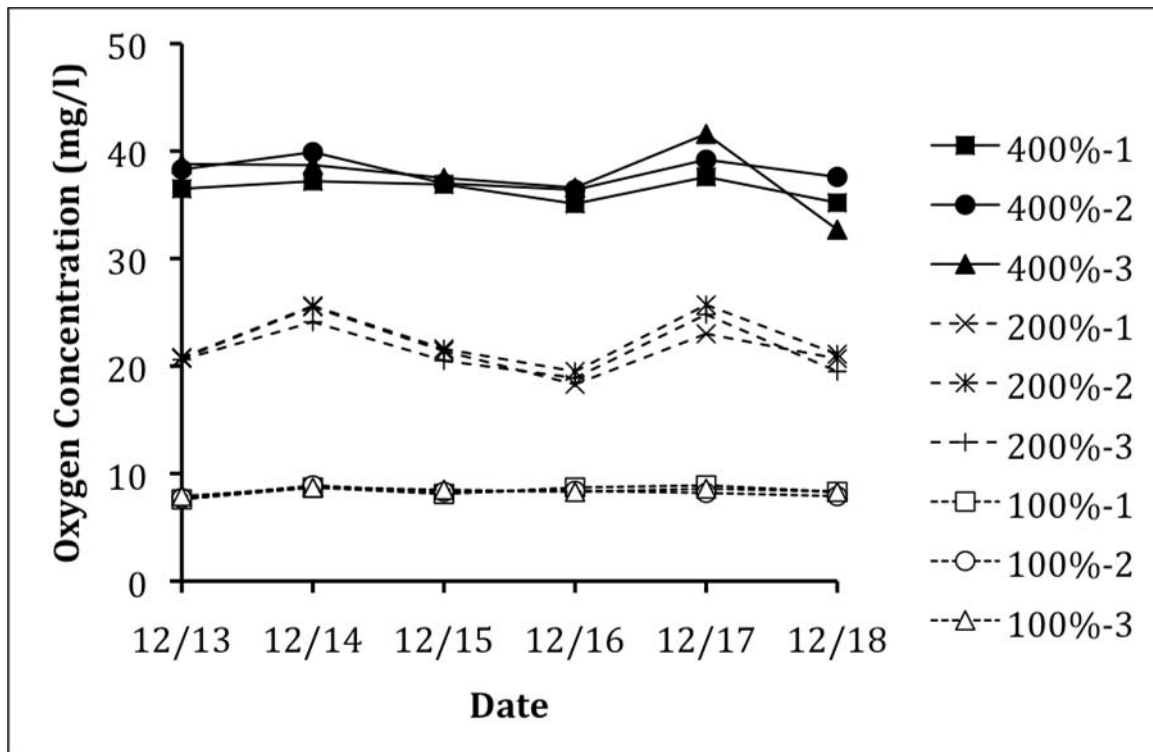


Figure 7. Polaris DO levels during the complete laboratory experiment.

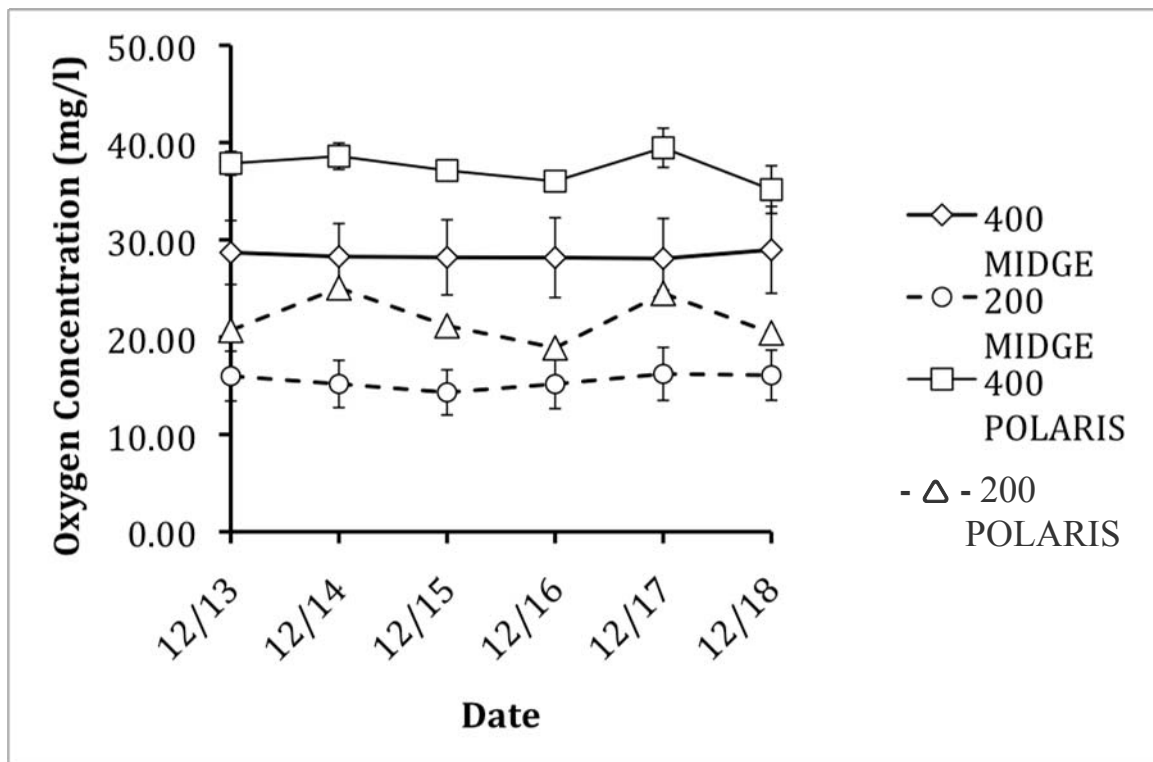


Figure 8. Comparison of Midge and Polaris DO readings during the complete laboratory experiment.

Growth of Juvenile Chinook Salmon

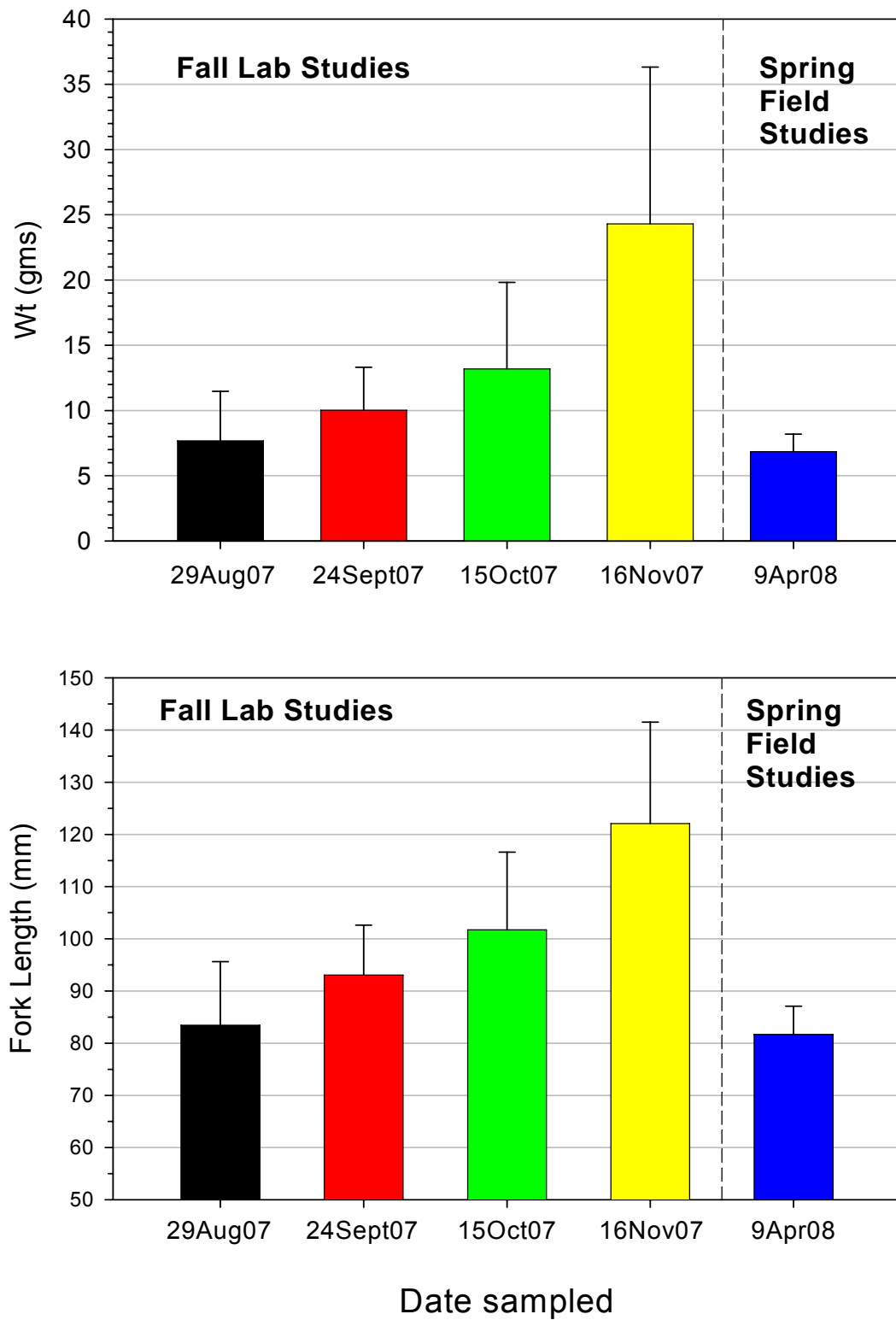


Figure 9. Fork length and weight (mean \pm s.d.) of juvenile Chinook salmon during acclimation for laboratory and field studies.

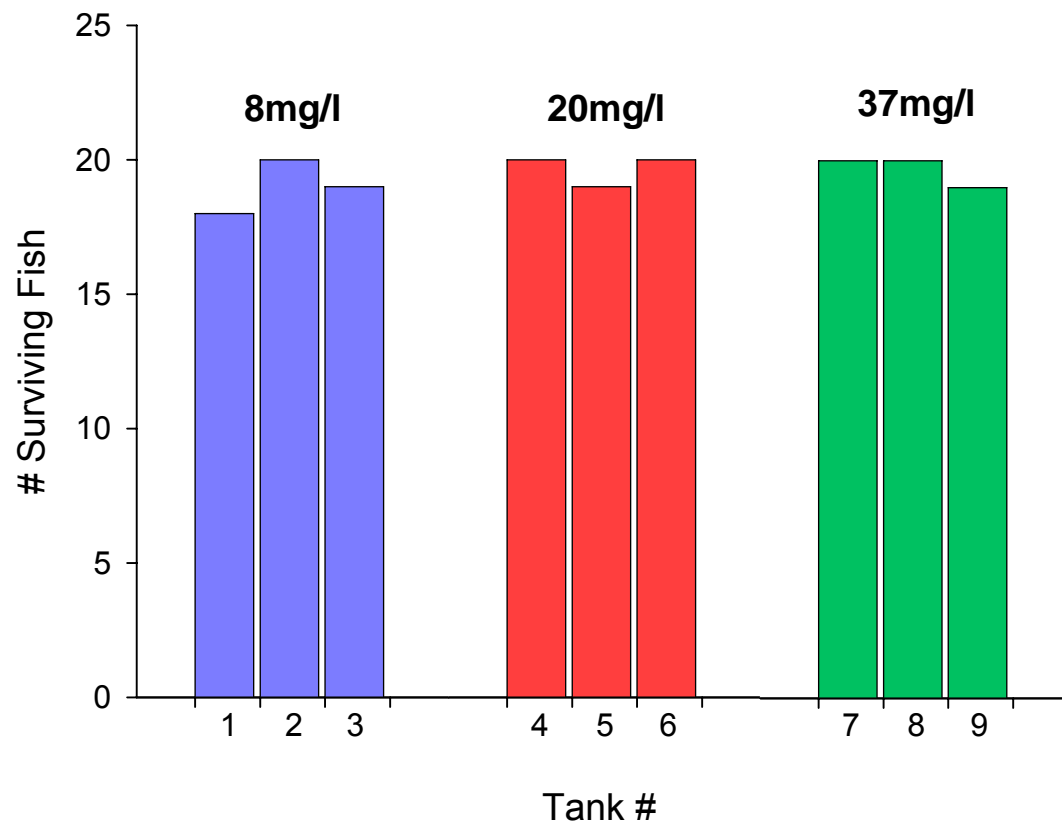


Figure 10. Saltwater challenge survival of hyperoxic treated fish from the complete laboratory experiment. Each replicate contained twenty fish. Salinity was rapidly elevated to 33psu and maintained for 24 hrs.

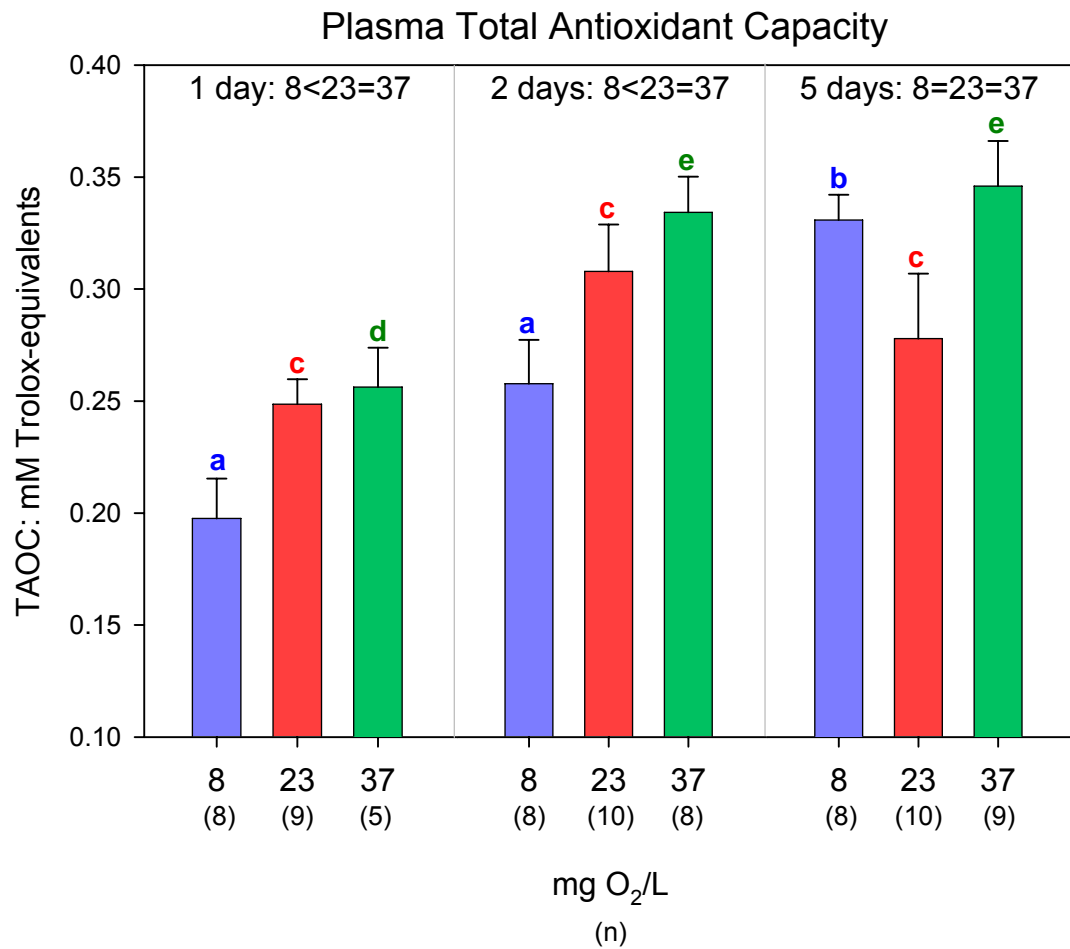


Figure 11. Plasma TAOC in juvenile salmon from the hyperoxic duration study. Bars represent mean \pm s.e. (n). A two-way ANOVA revealed significant differences. The difference in the mean values among the different levels of Days was greater than would be expected by chance after allowing for effects of differences in DO. And, the difference in the mean values among the different levels of DO was greater than would be expected by chance after allowing for effects of differences in Days. Aposteriori testing elucidated specific differences as described in Results.

Two-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Days	2	0.0913	0.0457	14.343	<0.001
DO	2	0.0318	0.0159	4.998	0.010
Days x DO	4	0.0252	0.00630	1.978	0.108
Residual	66	0.210	0.00318		
Total	74	0.369	0.00499		

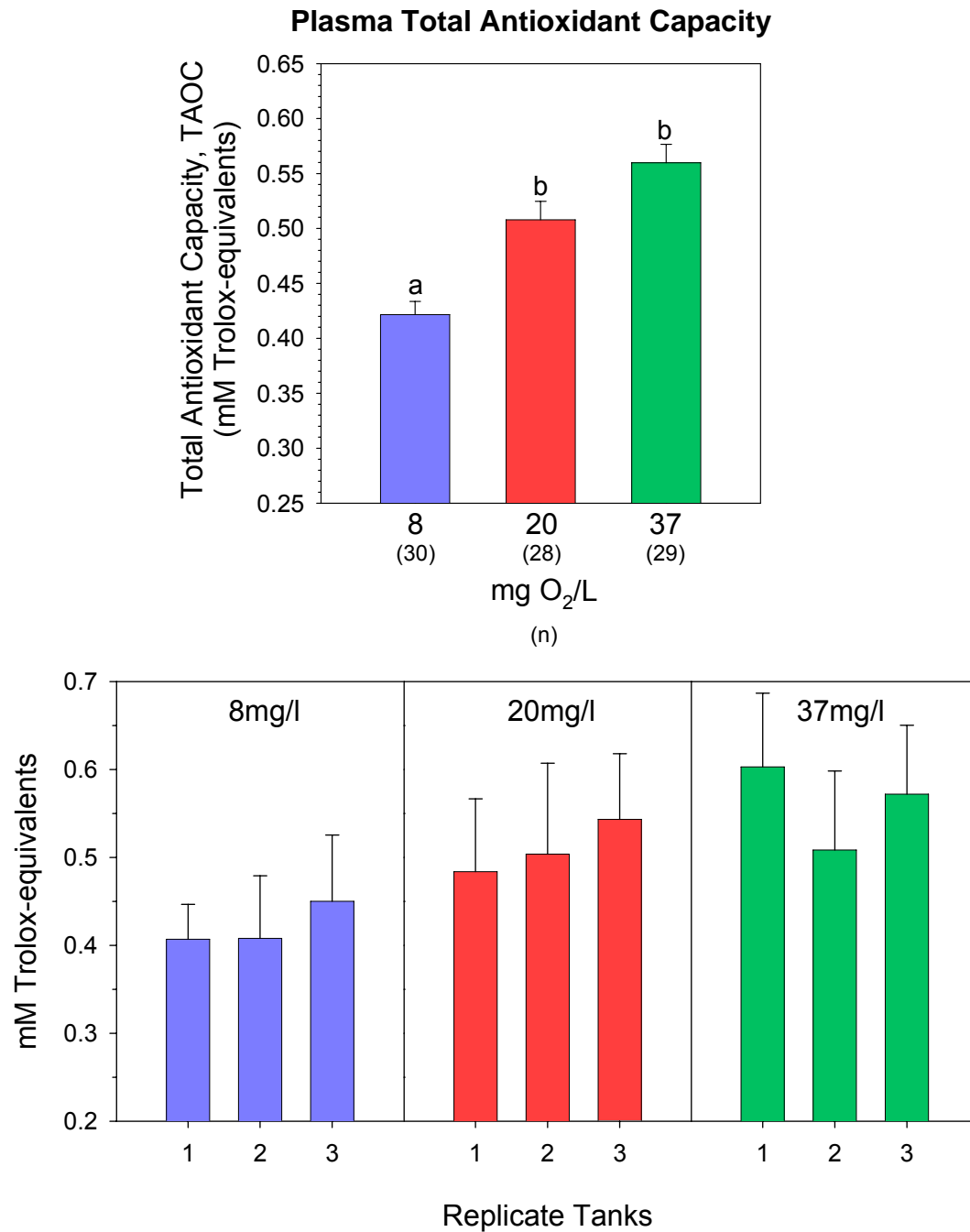


Figure 12. Plasma TAOC in juvenile salmon from the complete laboratory experiment. A nested ANOVA revealed no significant differences between replicates within each DO (bottom, $P=0.447$, mean \pm s.d.) but highly significant differences between DO levels (top, $P<0.001$, mean \pm s.e.(n)).

One-way Nested ANOVA Table

Source	df	SS	MS	F-ratio	P
DO	2	0.217	0.109	9.985	<0.001
Tanks(DO)	6	0.088	0.015	1.354	0.245
Error	71	0.773	0.011		

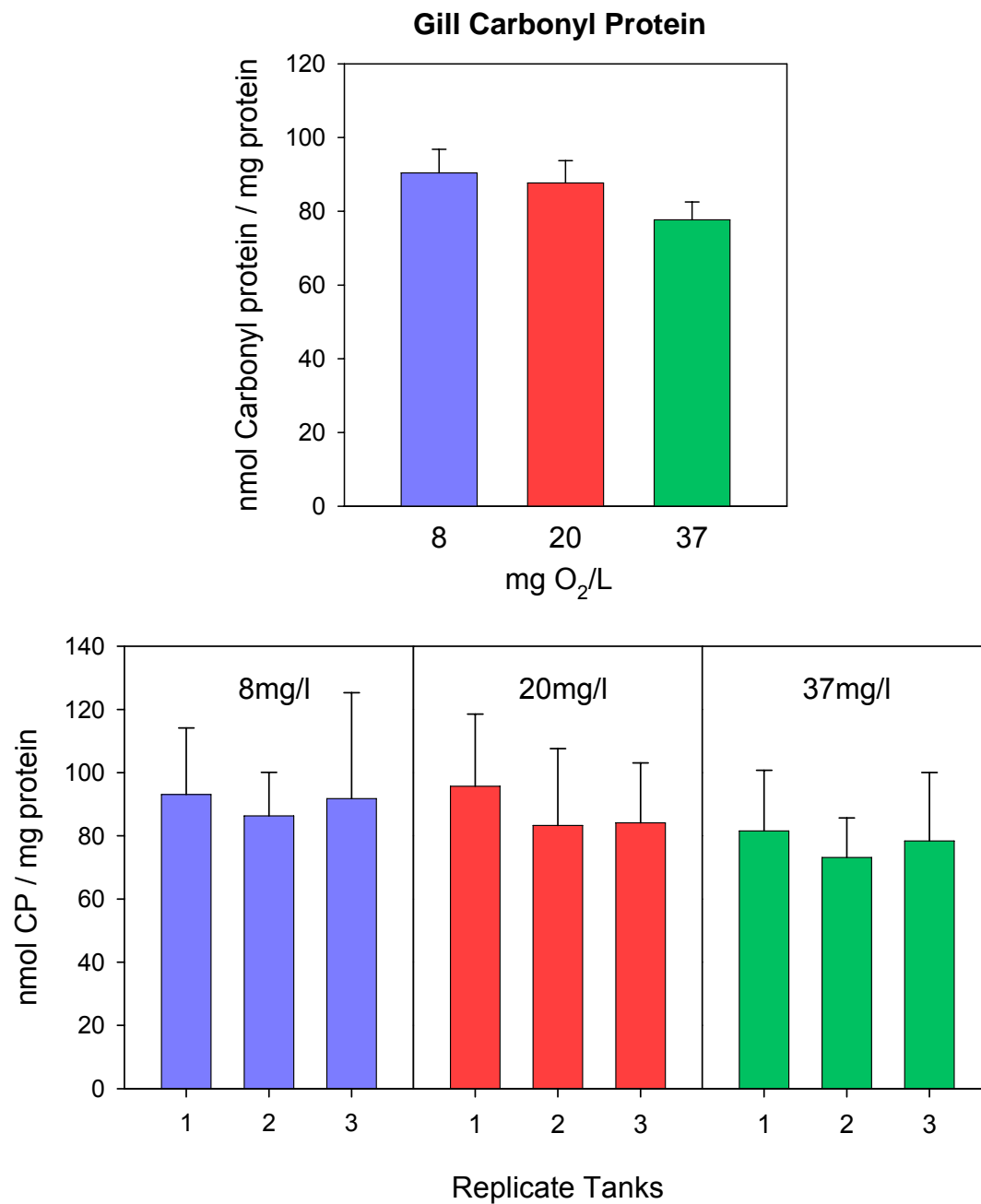


Fig. 13a. Carbonyl protein in Gill tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.986$, mean \pm s.d.) or between DO levels overall ($P=0.334$, mean \pm s.e., $n=12$) as determined by a one-way nested ANOVA.

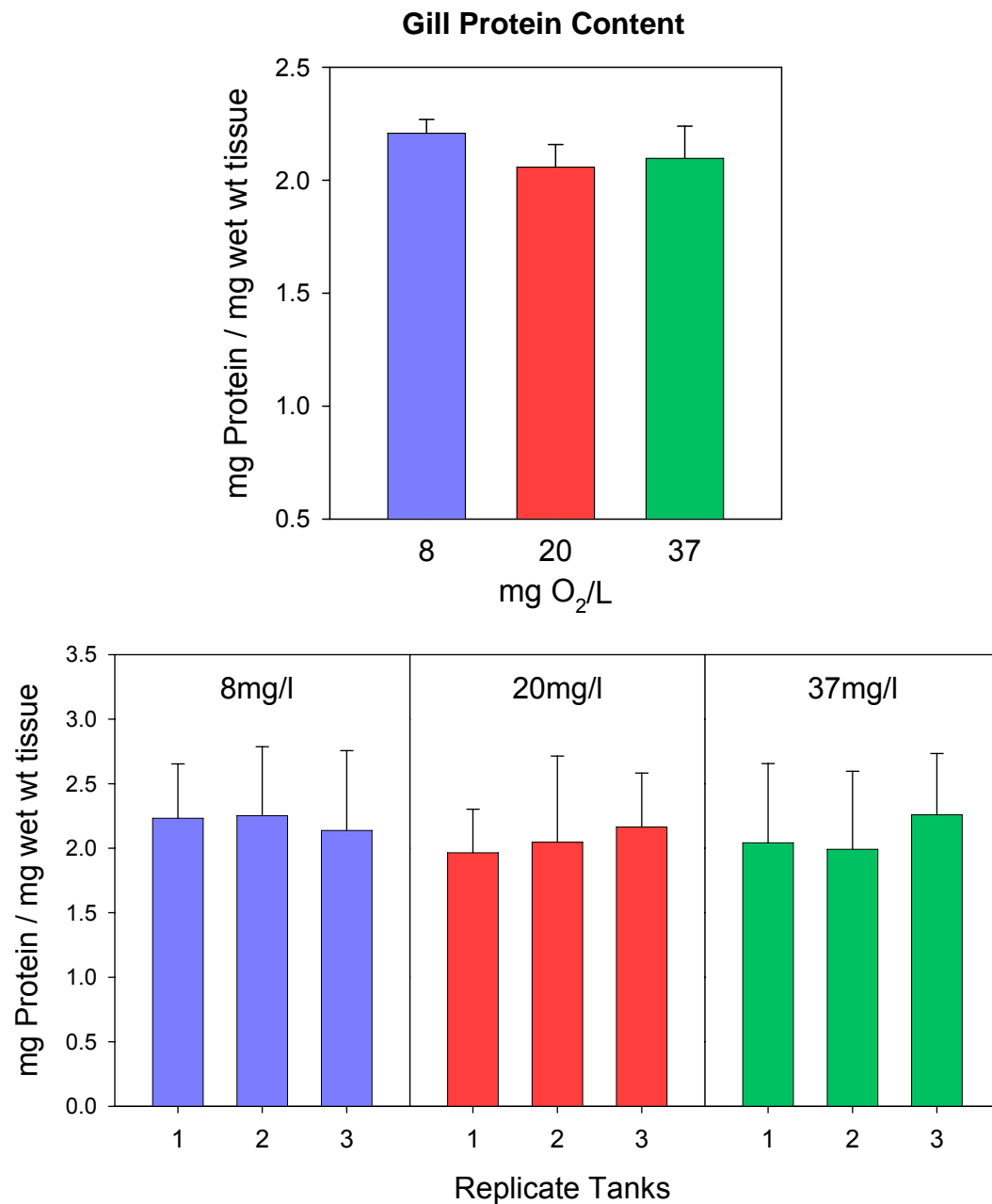


Figure 13b. Total protein in Gill tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.985$, mean \pm s.d.) or between DO levels overall ($P=0.778$, mean \pm s.e., $n=12$) as determined by a one-way nested ANOVA.

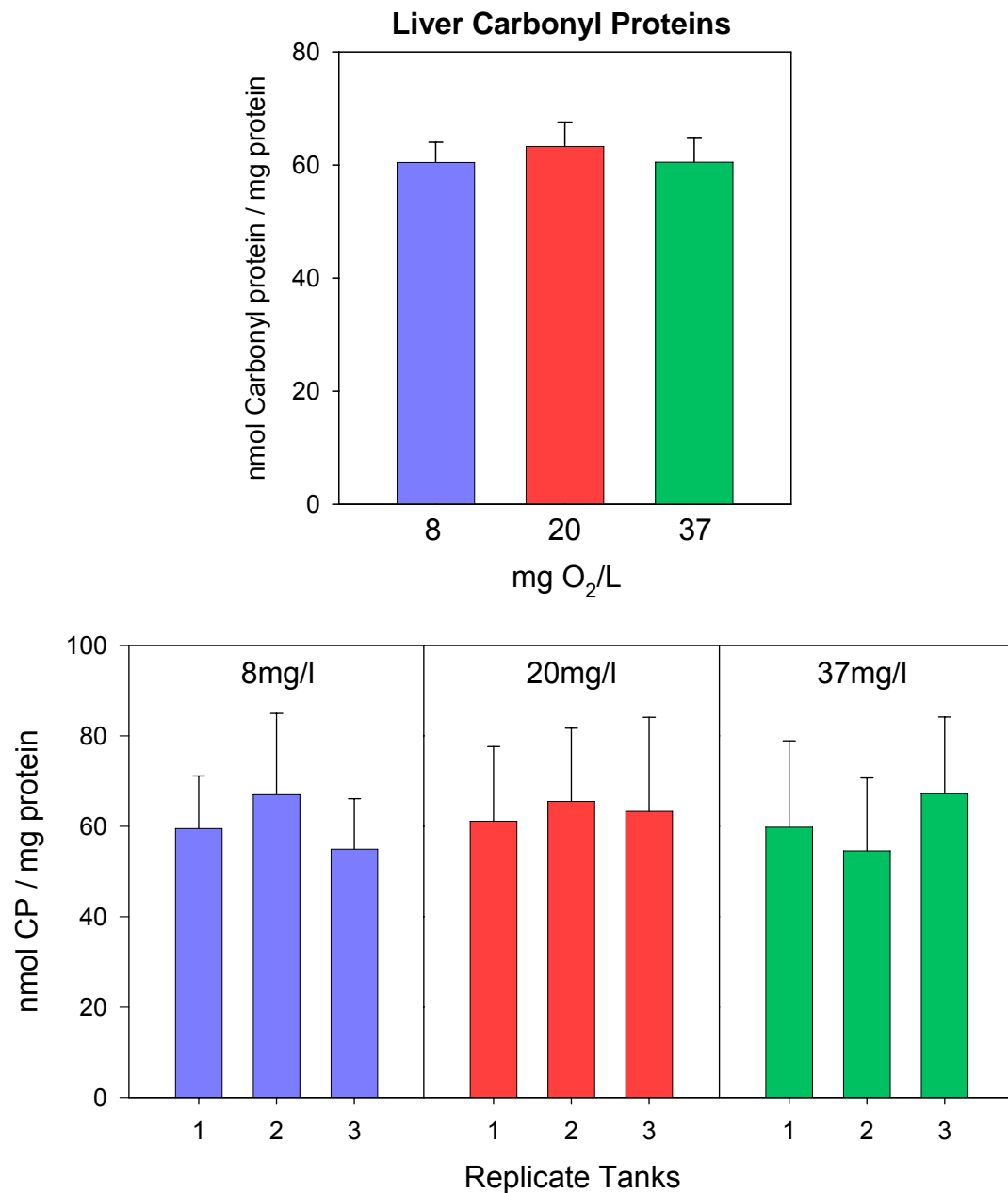


Figure 14a. Carbonyl protein in Liver tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.802$, mean \pm s.d.) or between DO levels overall ($P=0.867$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

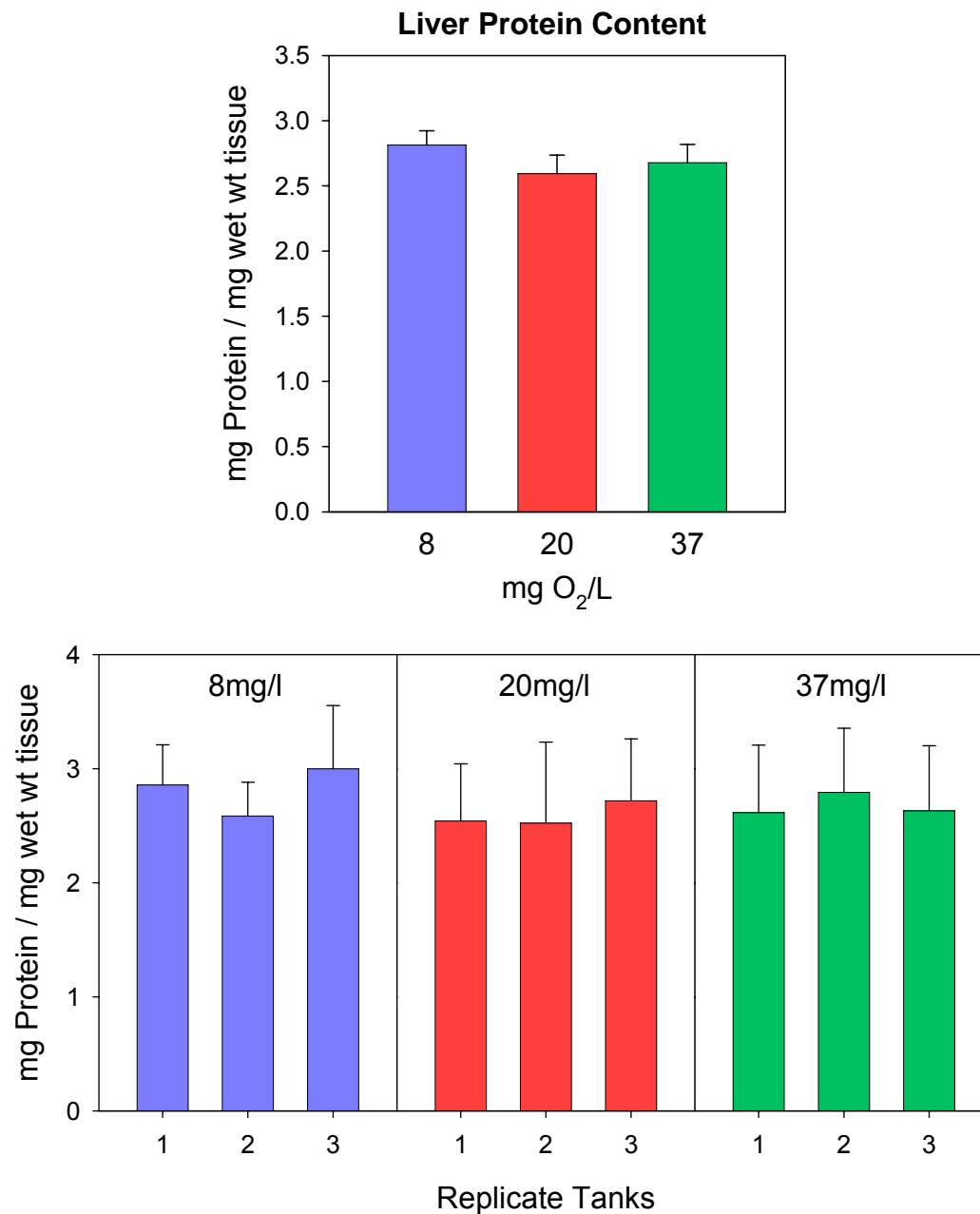


Figure 14b. Total protein in Liver tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.885$, mean \pm s.d.) or between DO levels overall ($P=0.529$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

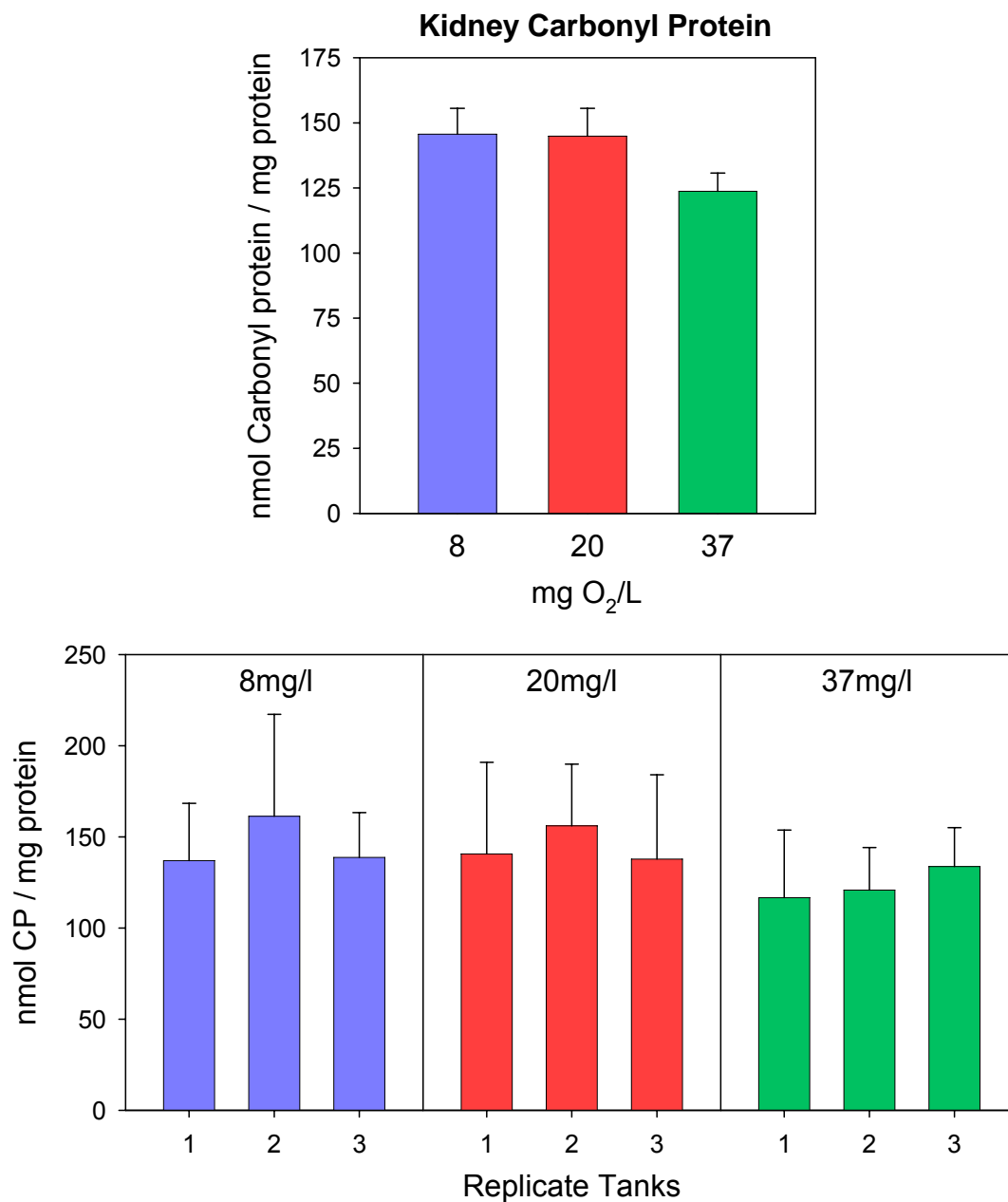


Figure 15a. Carbonyl protein in Kidney tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.860$, mean \pm s.d.) or between DO levels overall ($P=0.212$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

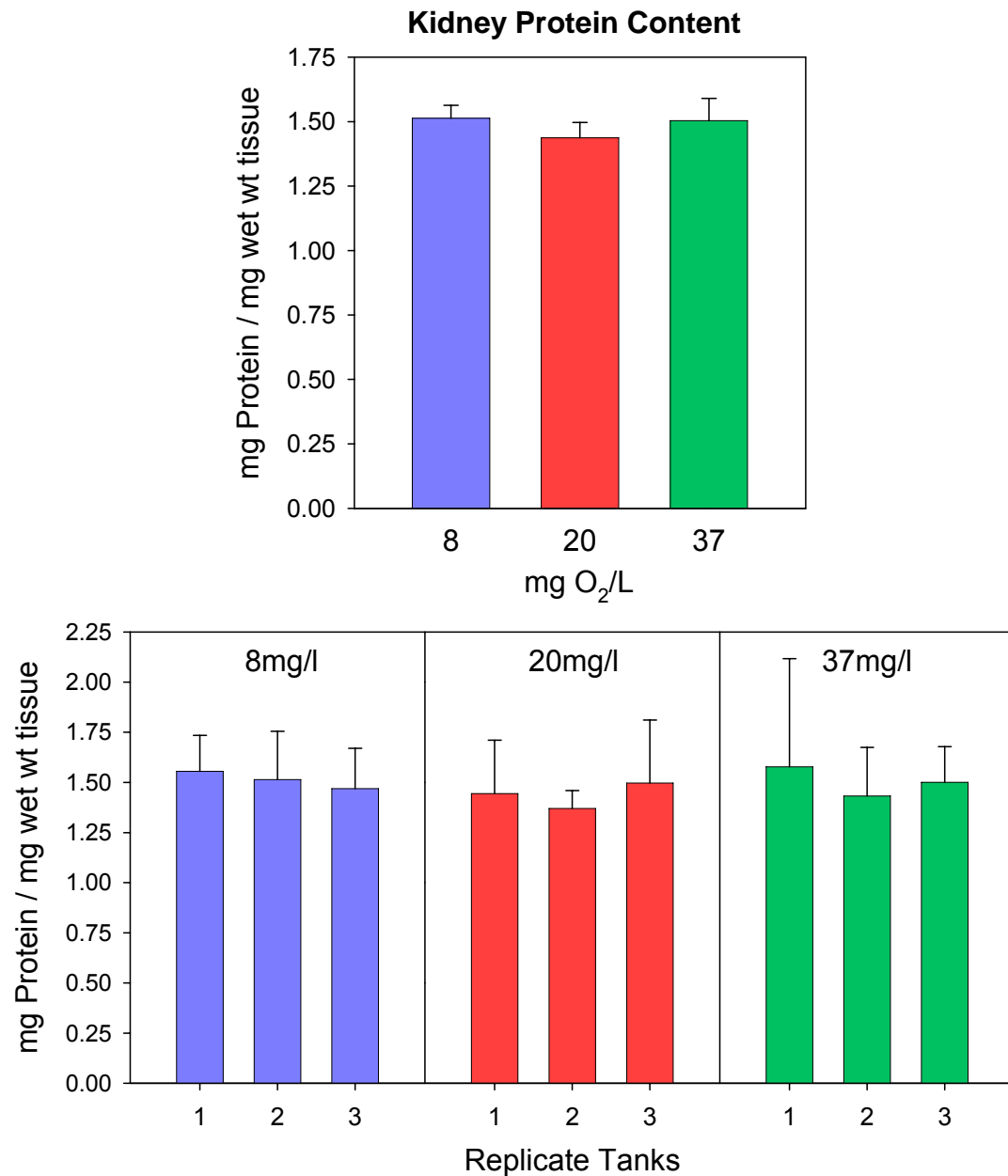


Figure 15b. Total protein in Kidney tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.958$, mean \pm s.d.) or between DO levels overall ($P=0.718$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

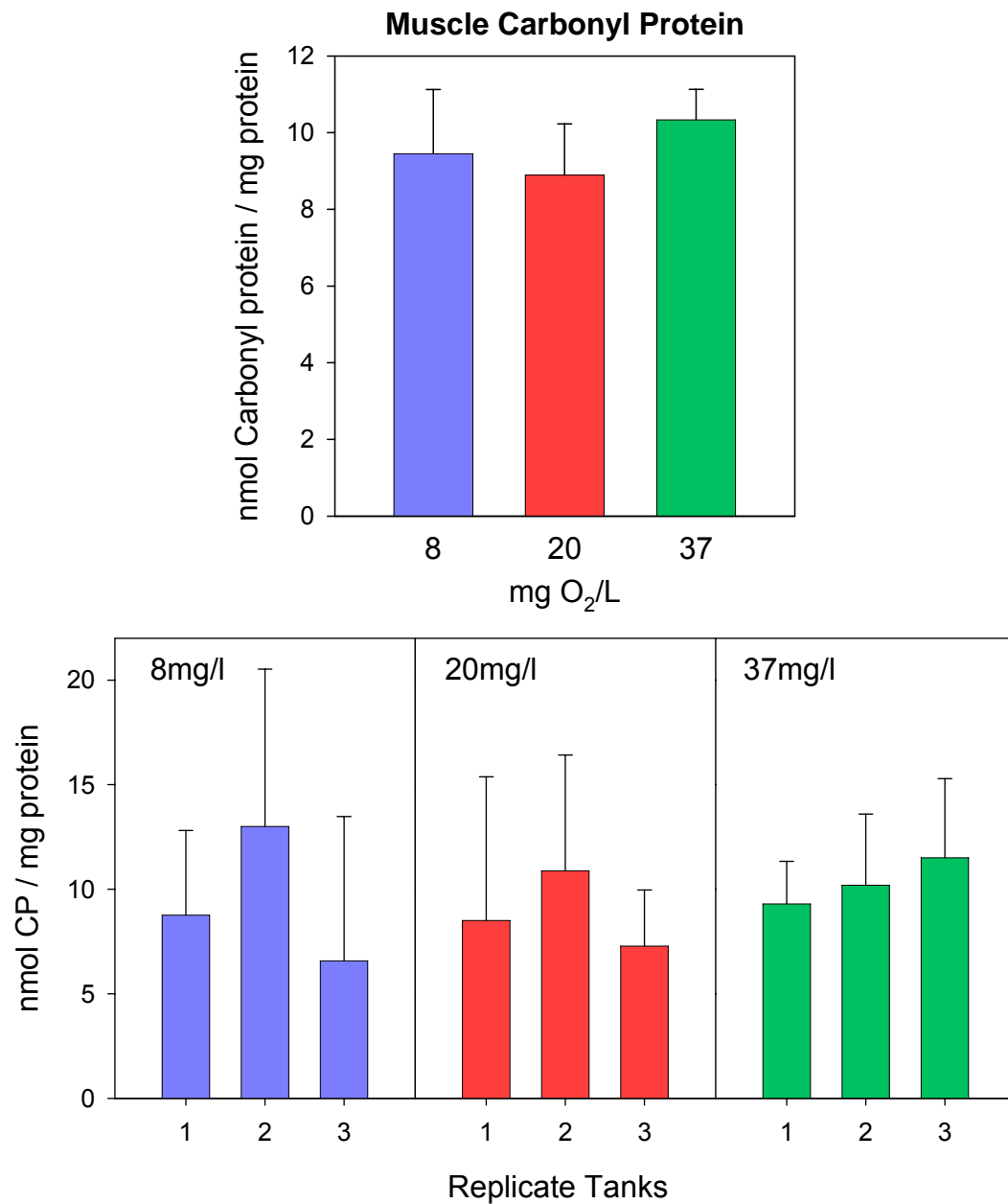


Figure 16a. Carbonyl protein in Muscle tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.458$, mean \pm s.d.) or between DO levels overall ($P=0.740$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

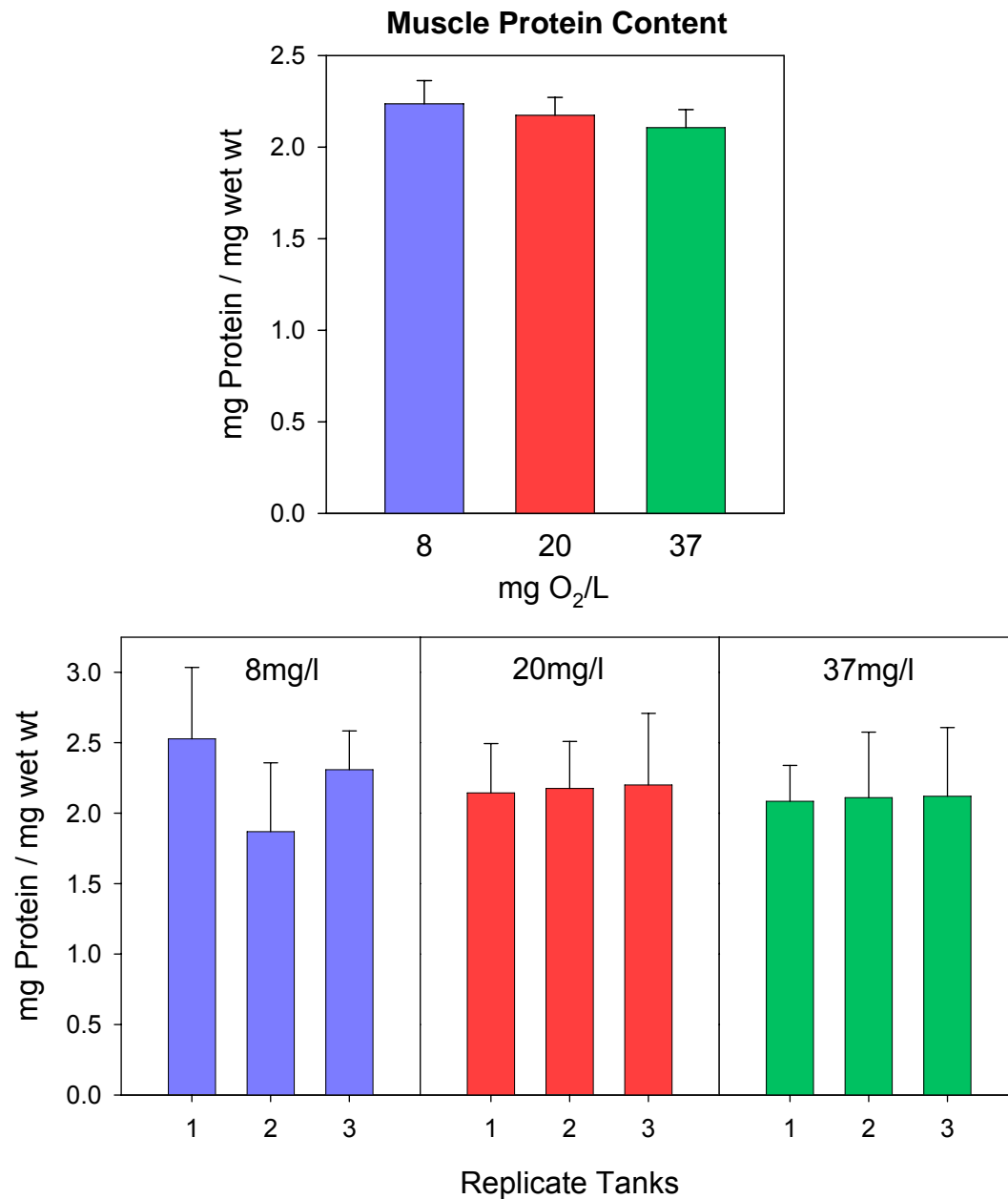


Figure 16b. Total protein in Muscle tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.394$, mean \pm s.d.) or between DO levels overall ($P=0.700$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

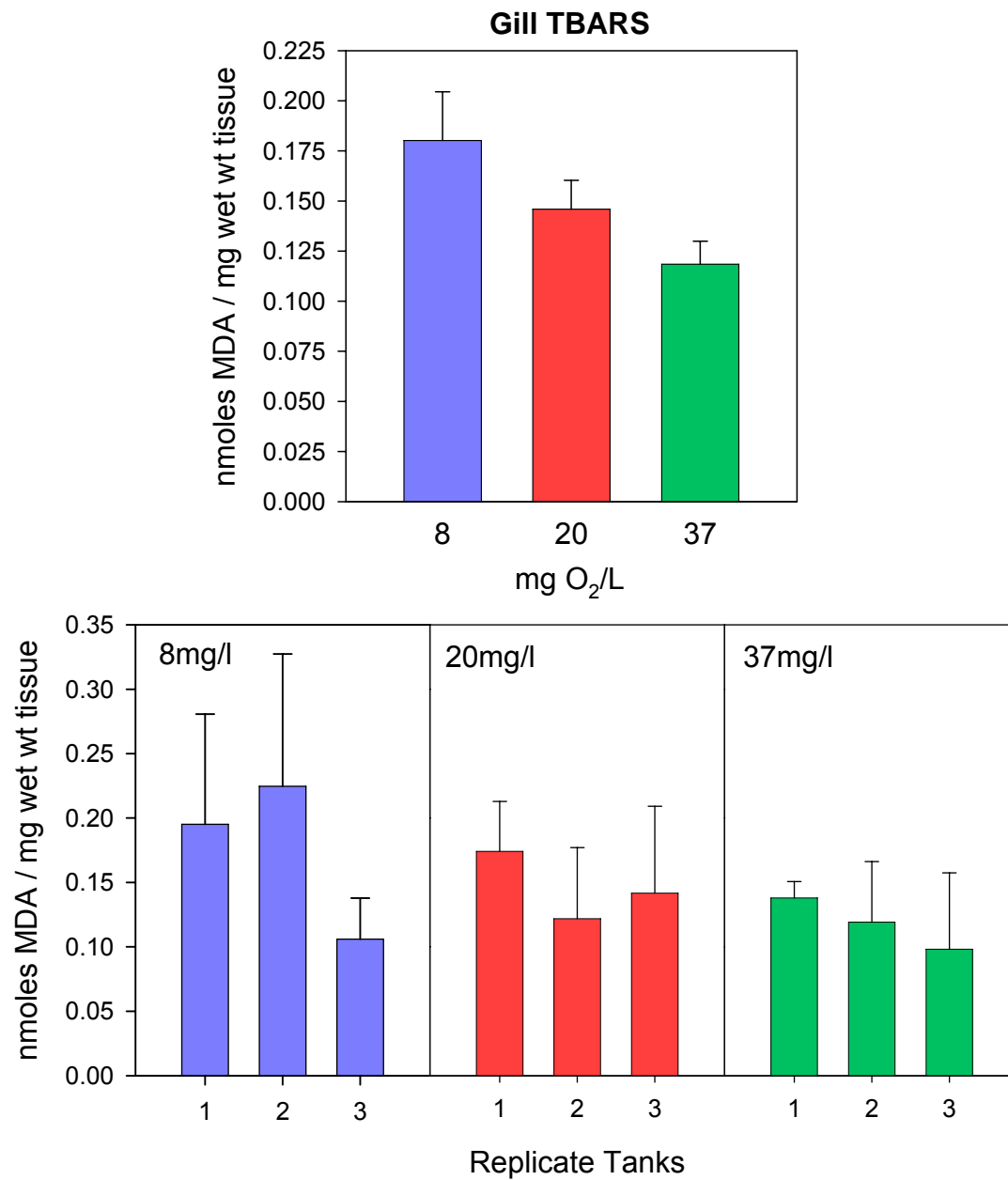


Figure 17a. Thiobarbituric Acid Reactive Substances (TBARS) in Gill tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.107$, mean \pm s.d.) or between DO levels overall ($P=0.061$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

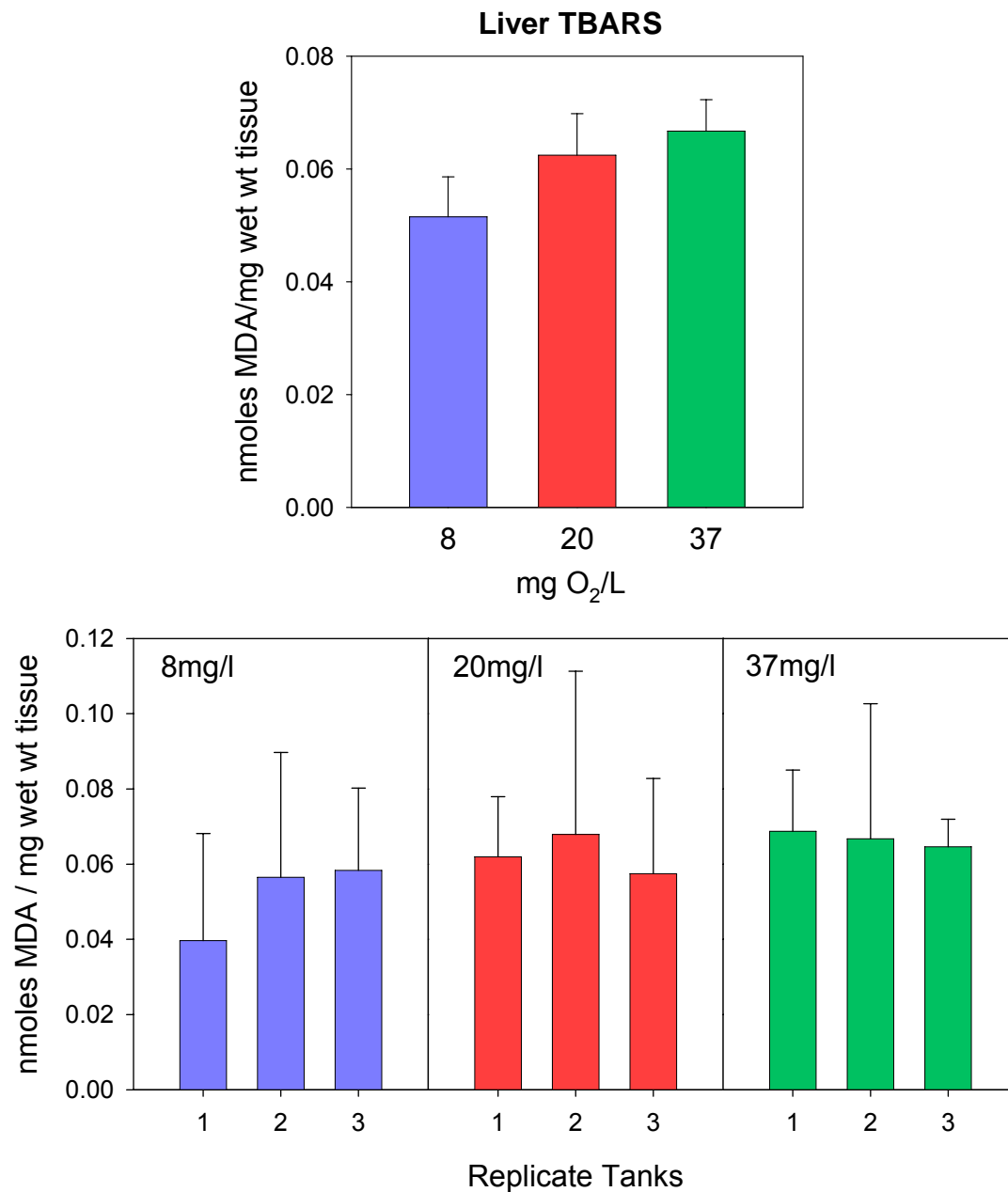


Figure 17b. Thiobarbituric Acid Reactive Substances (TBARS) in Liver tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.928$, mean \pm s.d.) or between DO levels overall ($P=0.304$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

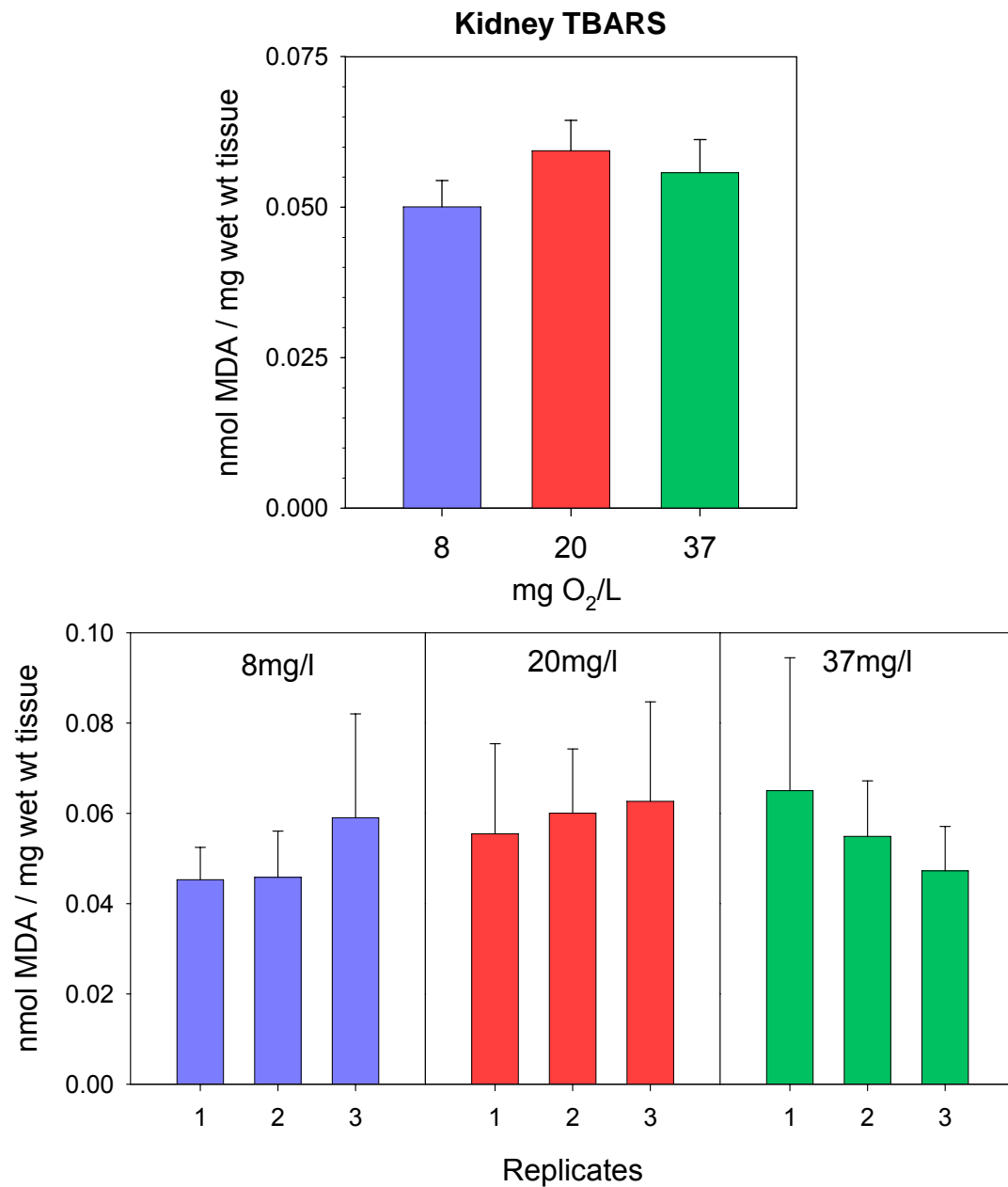


Figure 17c. Thiobarbituric Acid Reactive Substances (TBARS) in Kidney tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.699$, mean \pm s.d.) or between DO levels overall ($P=0.450$, mean \pm s.e., $n=12$) as determined by a one-way nested ANOVA.

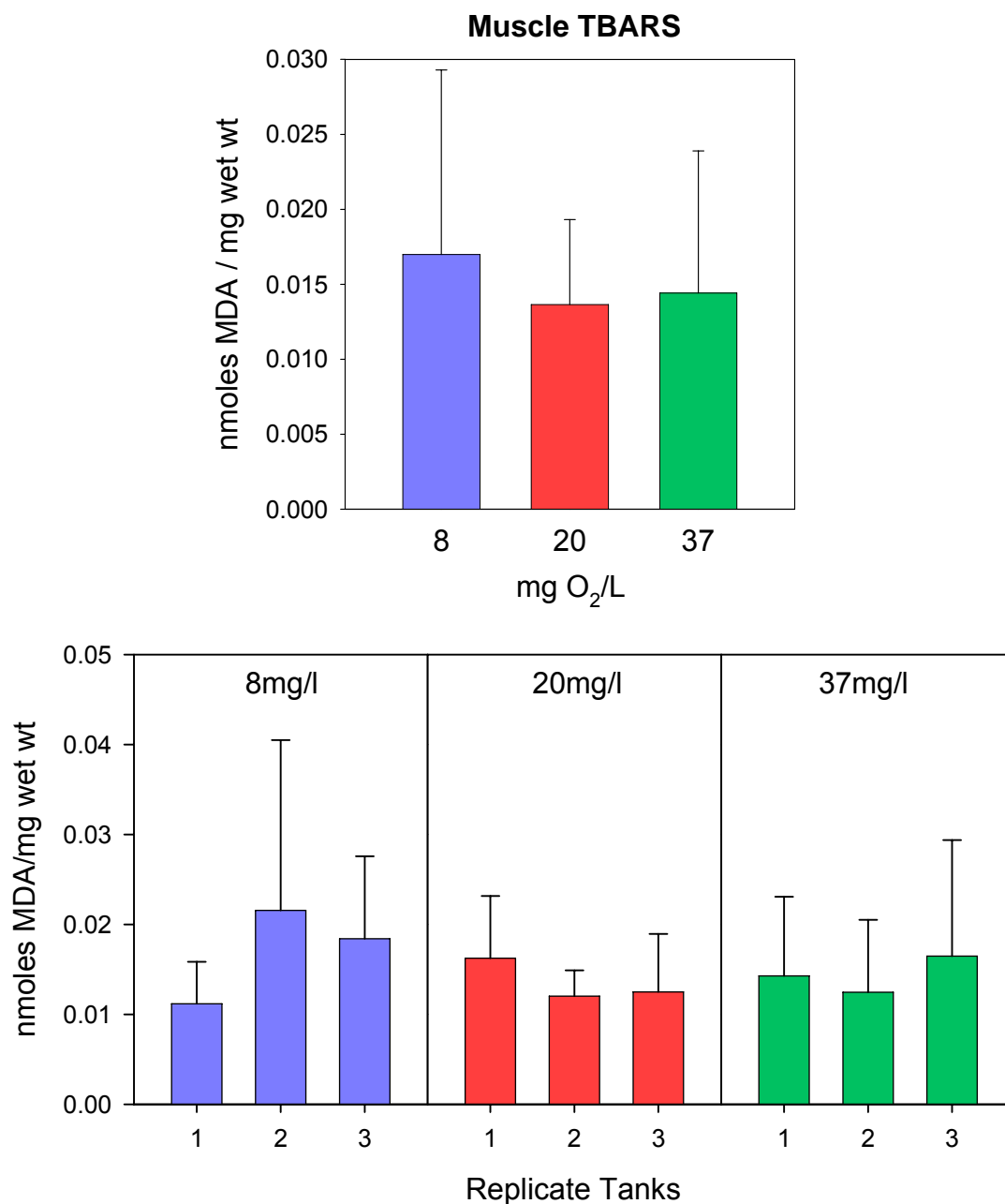


Figure 17d. Thiobarbituric Acid Reactive Substances (TBARS) in Muscle tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.685$, mean \pm s.d.) or between DO levels overall ($P=0.605$, mean \pm s.e., $n=12$) as determined by a one-way nested ANOVA.

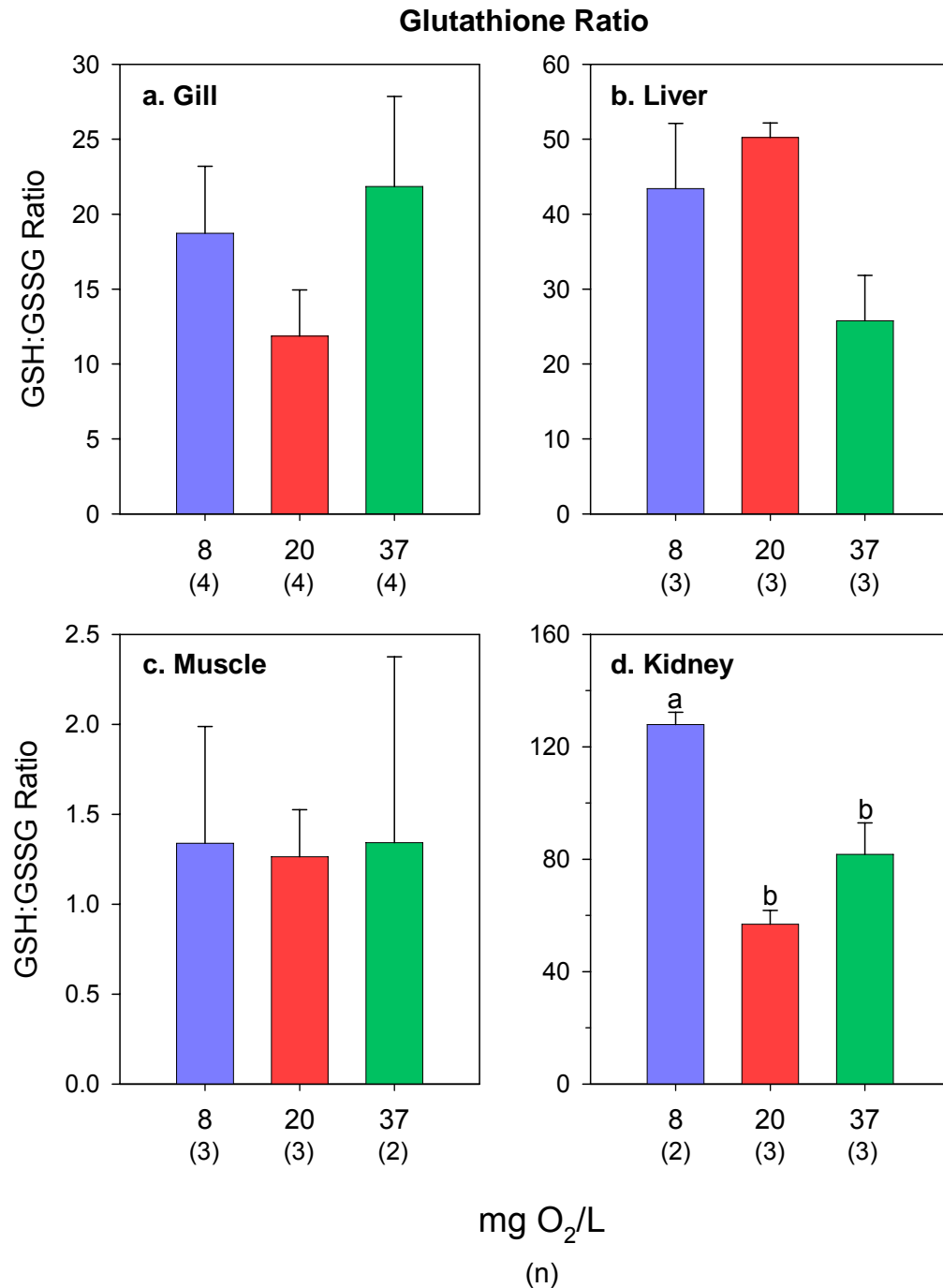


Figure 18. Glutathione ratios in a. Gill, b. Liver, c. Muscle, and d. Kidney tissue from fish in the preliminary experiment (mean \pm s.e., (n)). There were no significant differences between DO levels in gill ($P=0.346$), liver ($P=0.075$), or muscle ($P=0.439$) based on a one-way ANOVA. In kidney, elevated DO (20 & 37mg/l) saw a significant reduction in glutathione ratio, i.e. oxidized GSSG went up relative to reduced GSH ($P=0.007$).

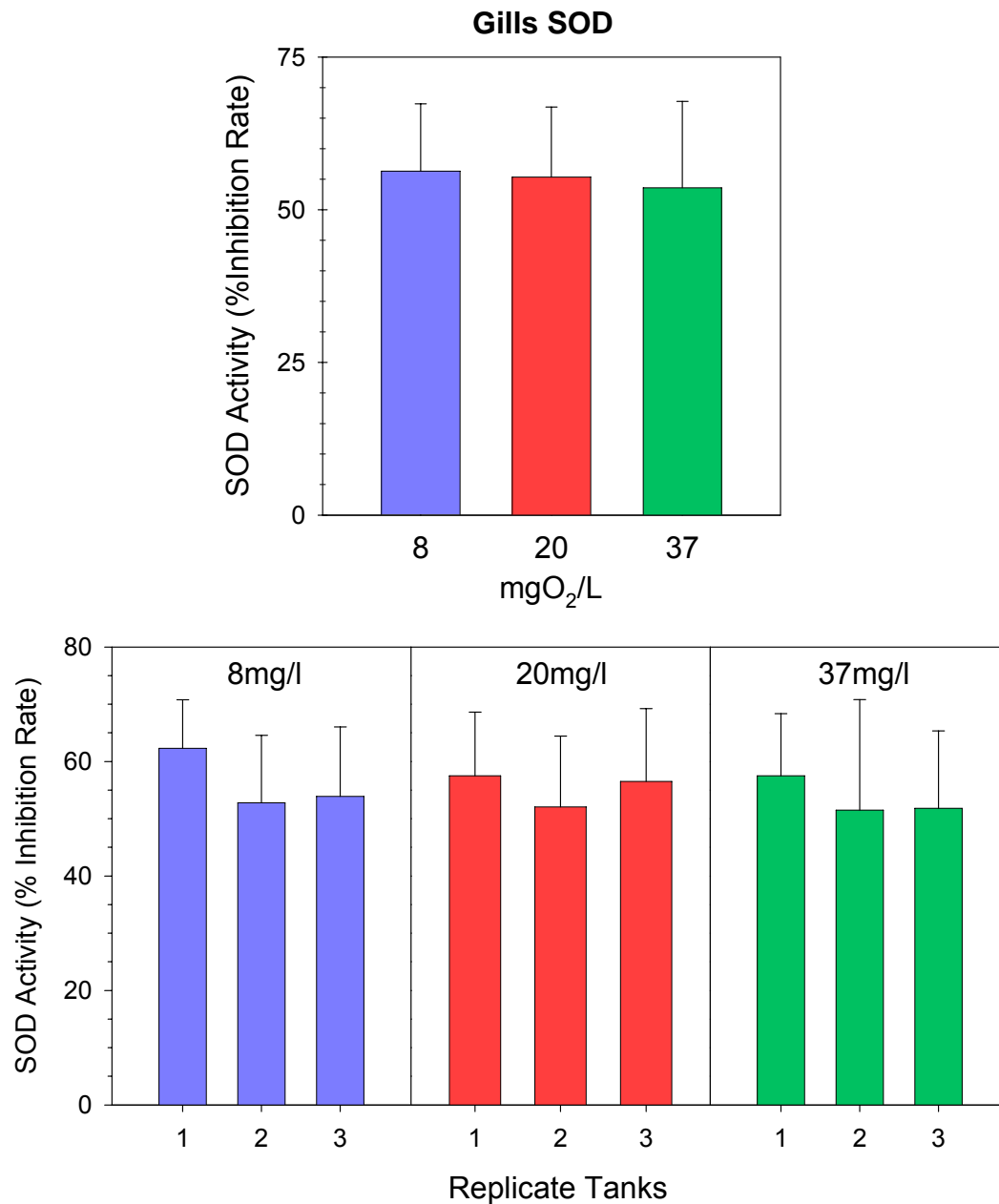


Figure 19a. SOD activity in Gill tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.820$, mean \pm s.d.) or between DO levels overall ($P=0.842$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

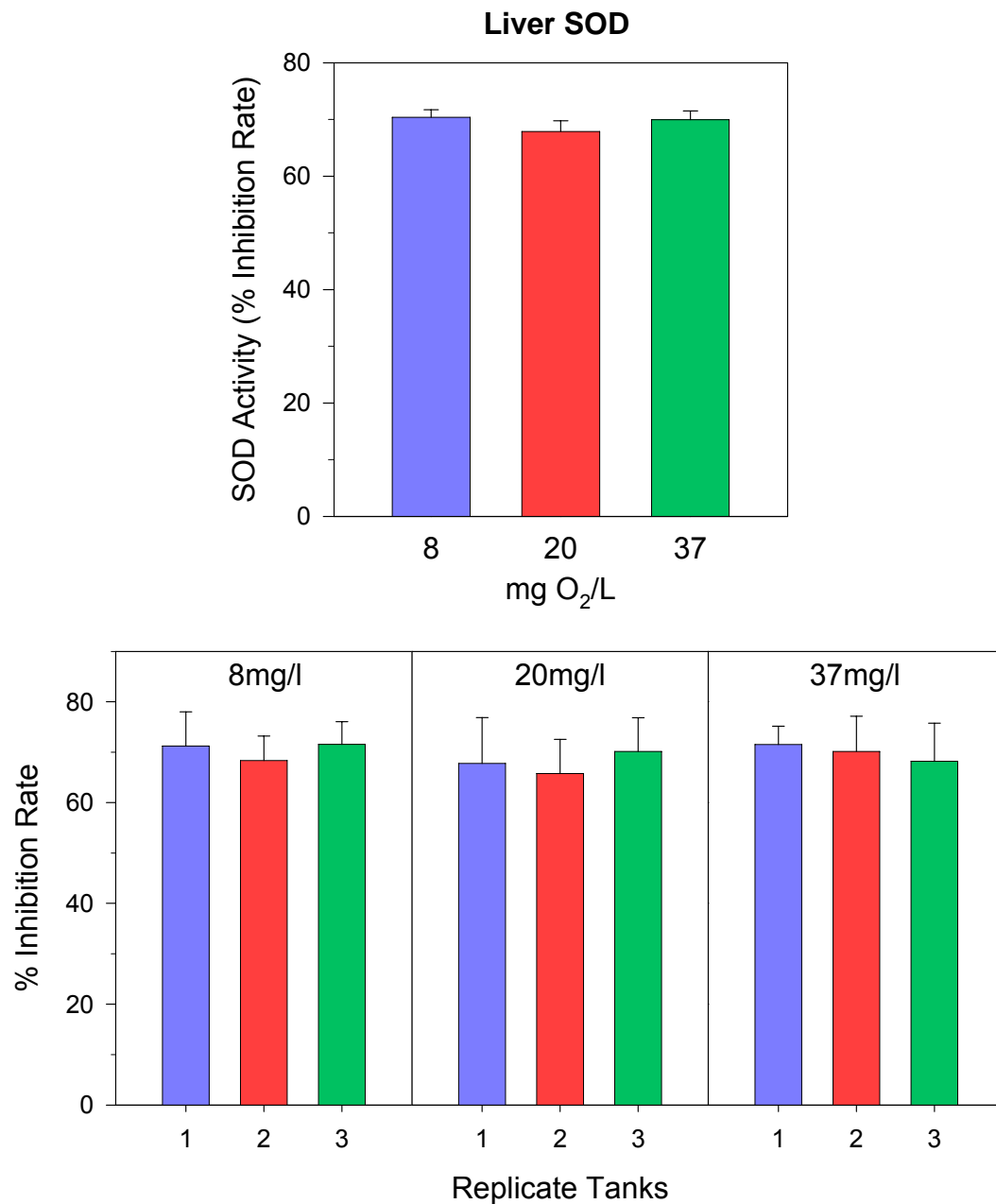


Figure 19b. SOD activity in Liver tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.861$, mean \pm s.d.) or between DO levels overall ($P=0.542$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

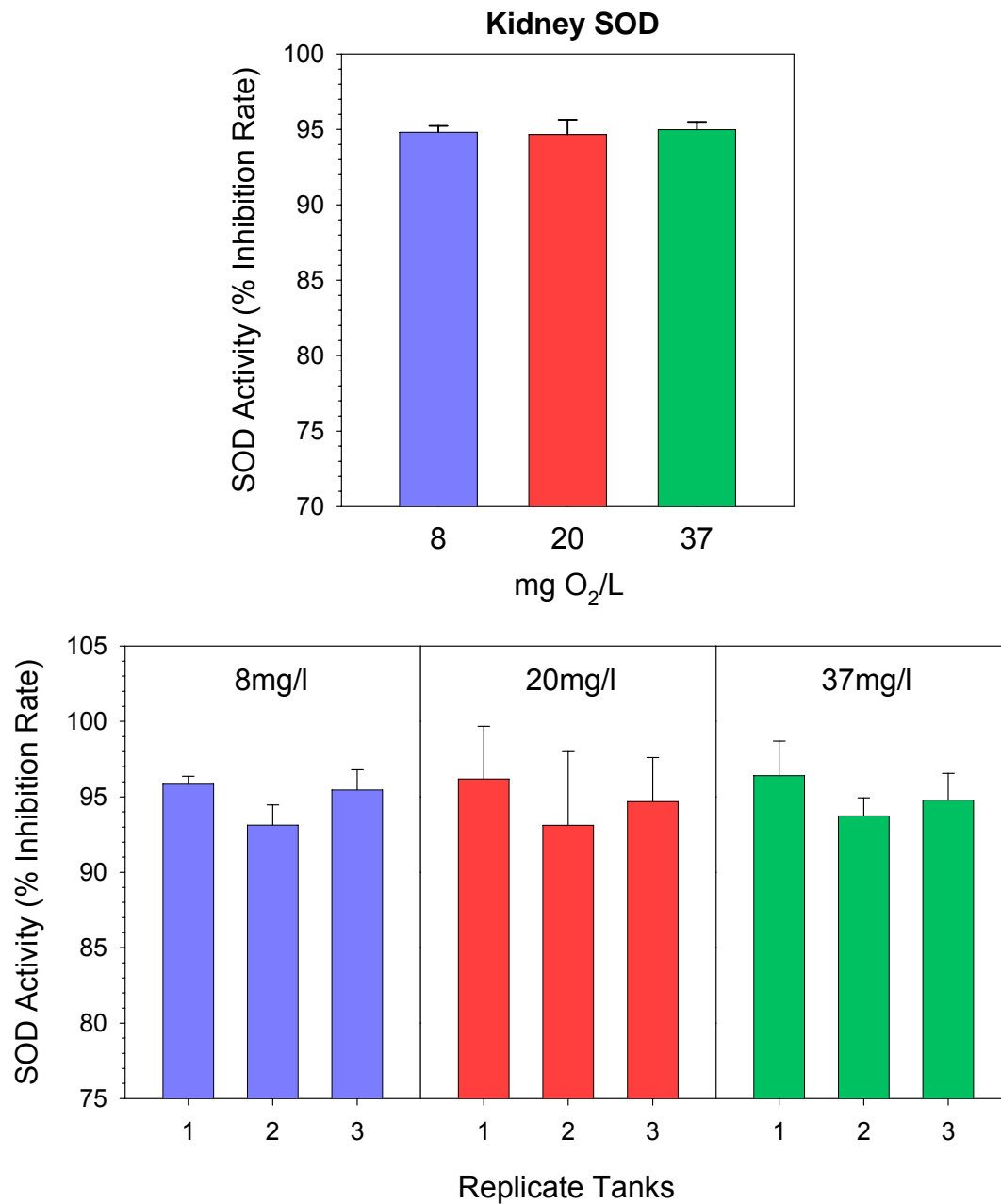


Figure 19c. SOD activity in Kidney tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.170$, mean \pm s.d.) or between DO levels overall ($P=0.944$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

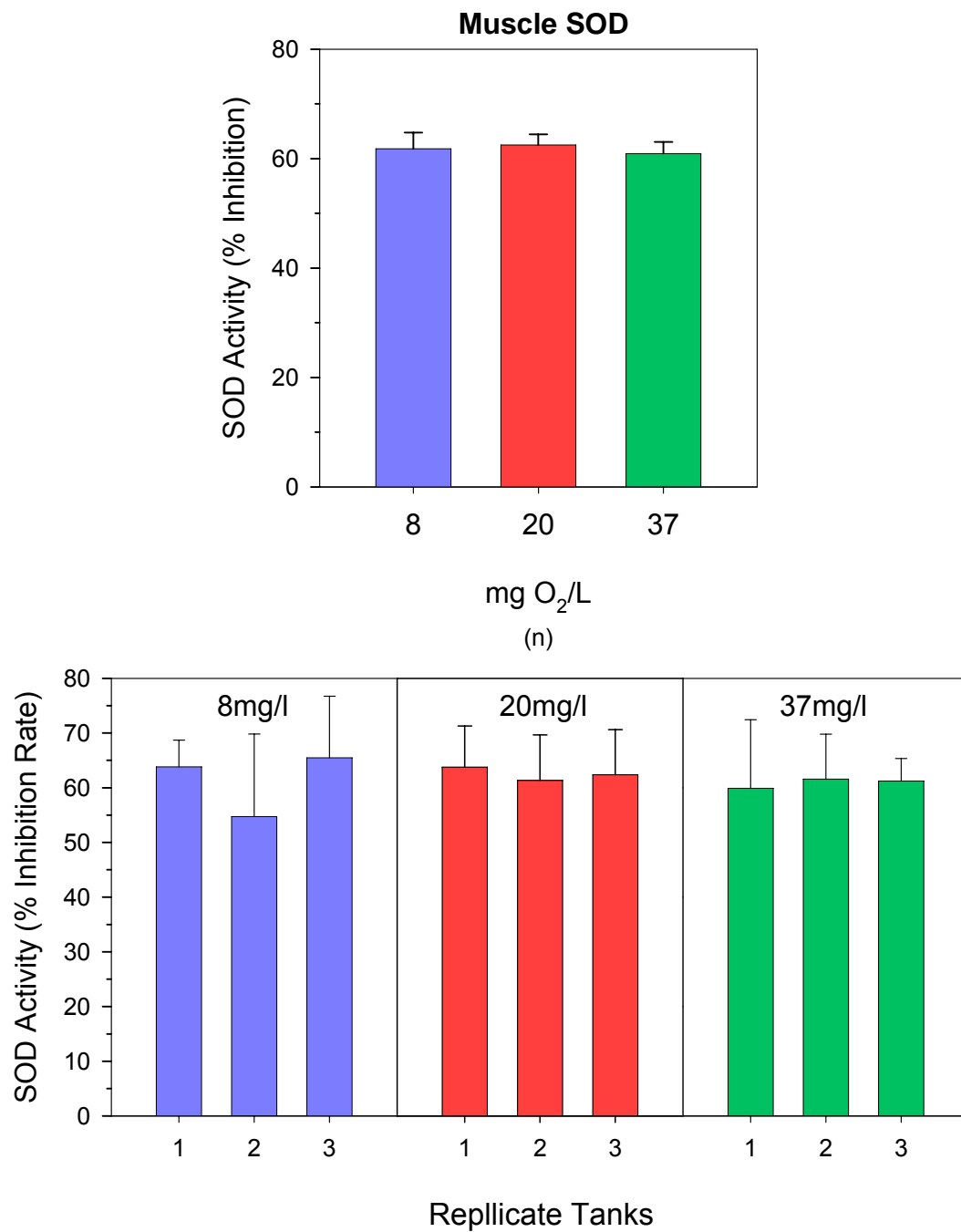


Figure 19d. SOD activity in Muscle tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.726$, mean \pm s.d.) or between DO levels overall ($P=0.889$, mean \pm s.e.(n)) as determined by a one-way nested ANOVA.

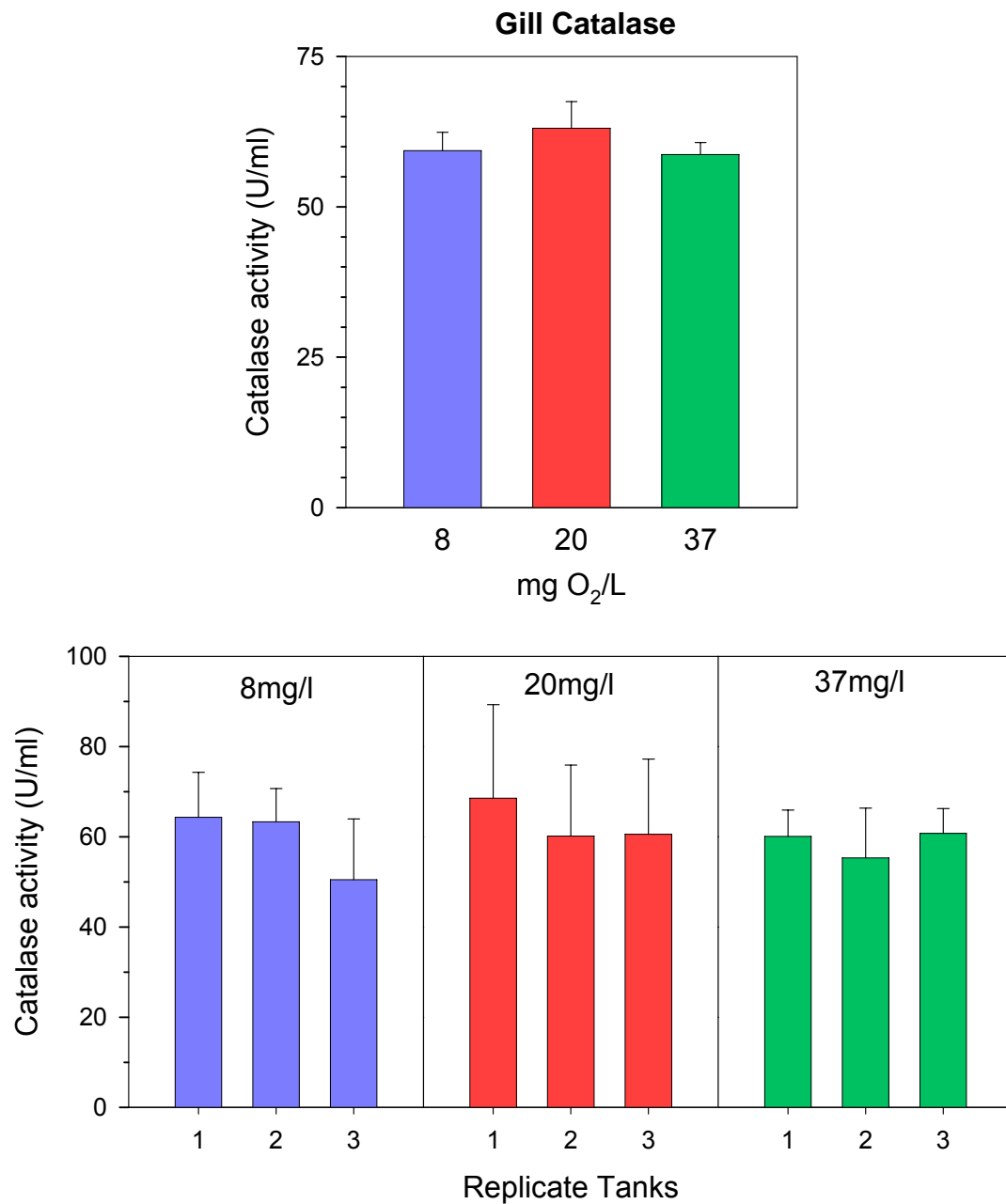


Figure 20a. Catalase activity in Gill tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.491$, mean \pm s.d.) or between DO levels overall ($P=0.603$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

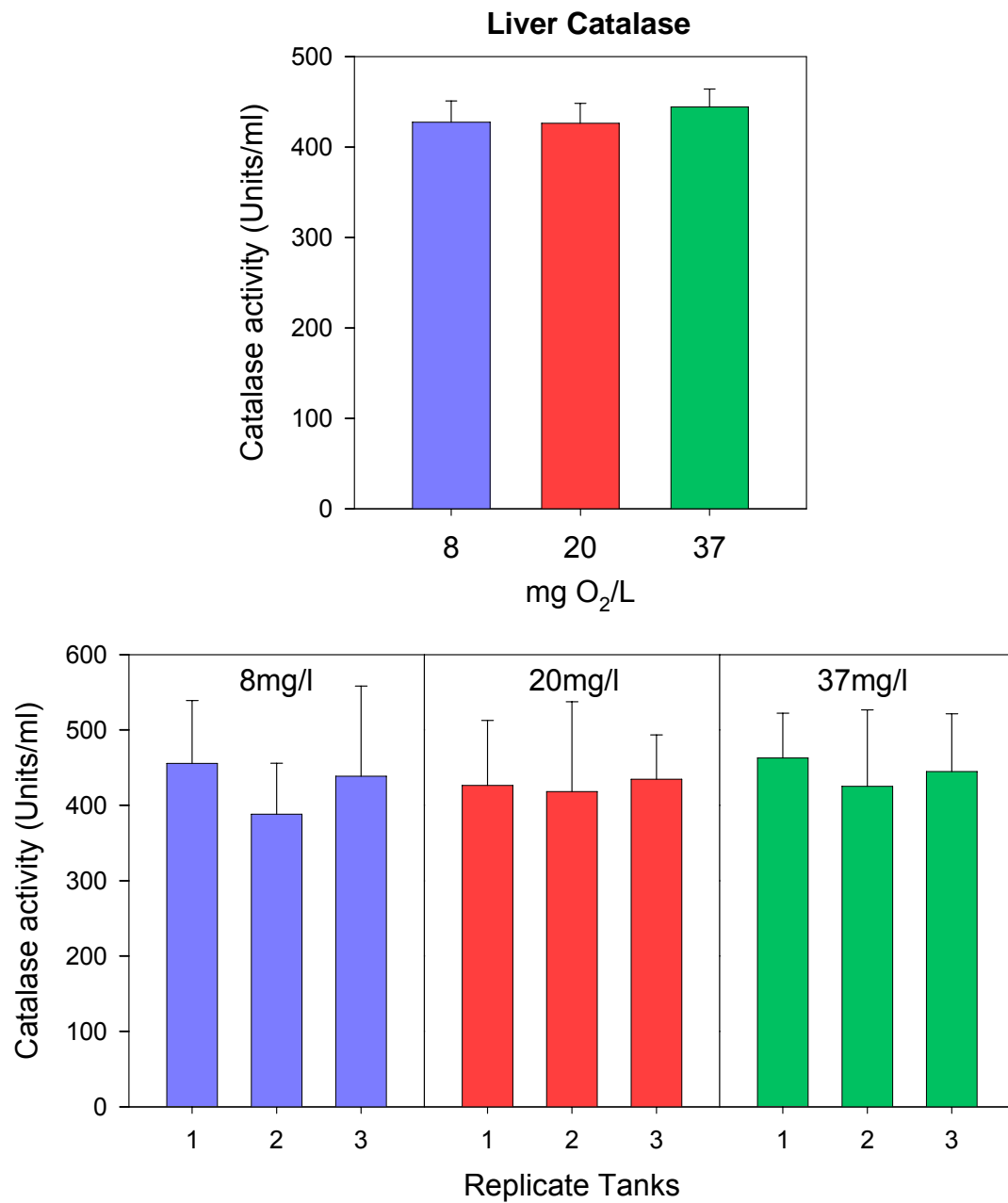


Figure 20b. Catalase activity in Liver tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.904$, mean \pm s.d.) or between DO levels overall ($P=0.825$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

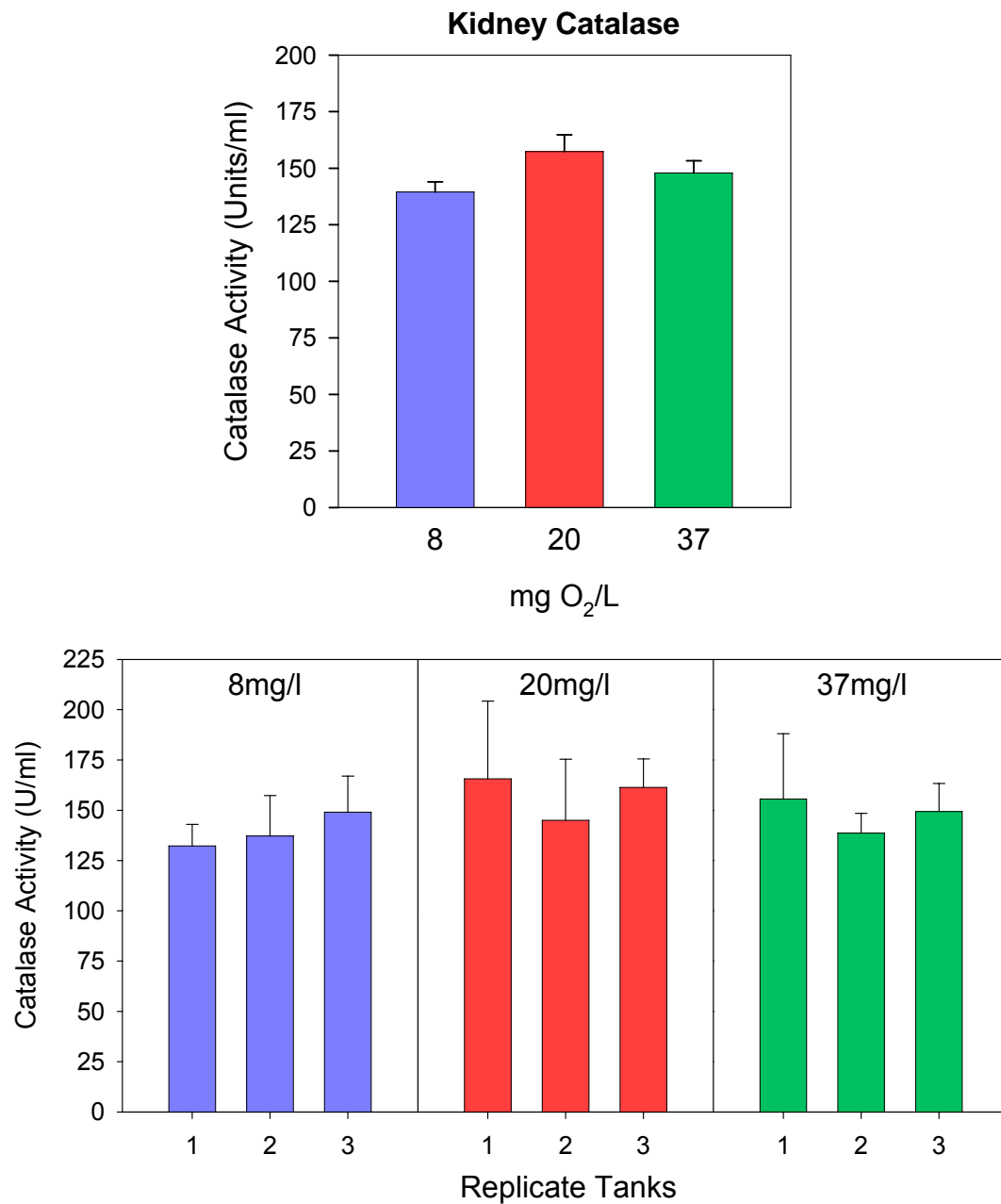


Figure 20c. Catalase activity in Kidney tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.552$, mean \pm s.d.) or between DO levels overall ($P=0.121$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

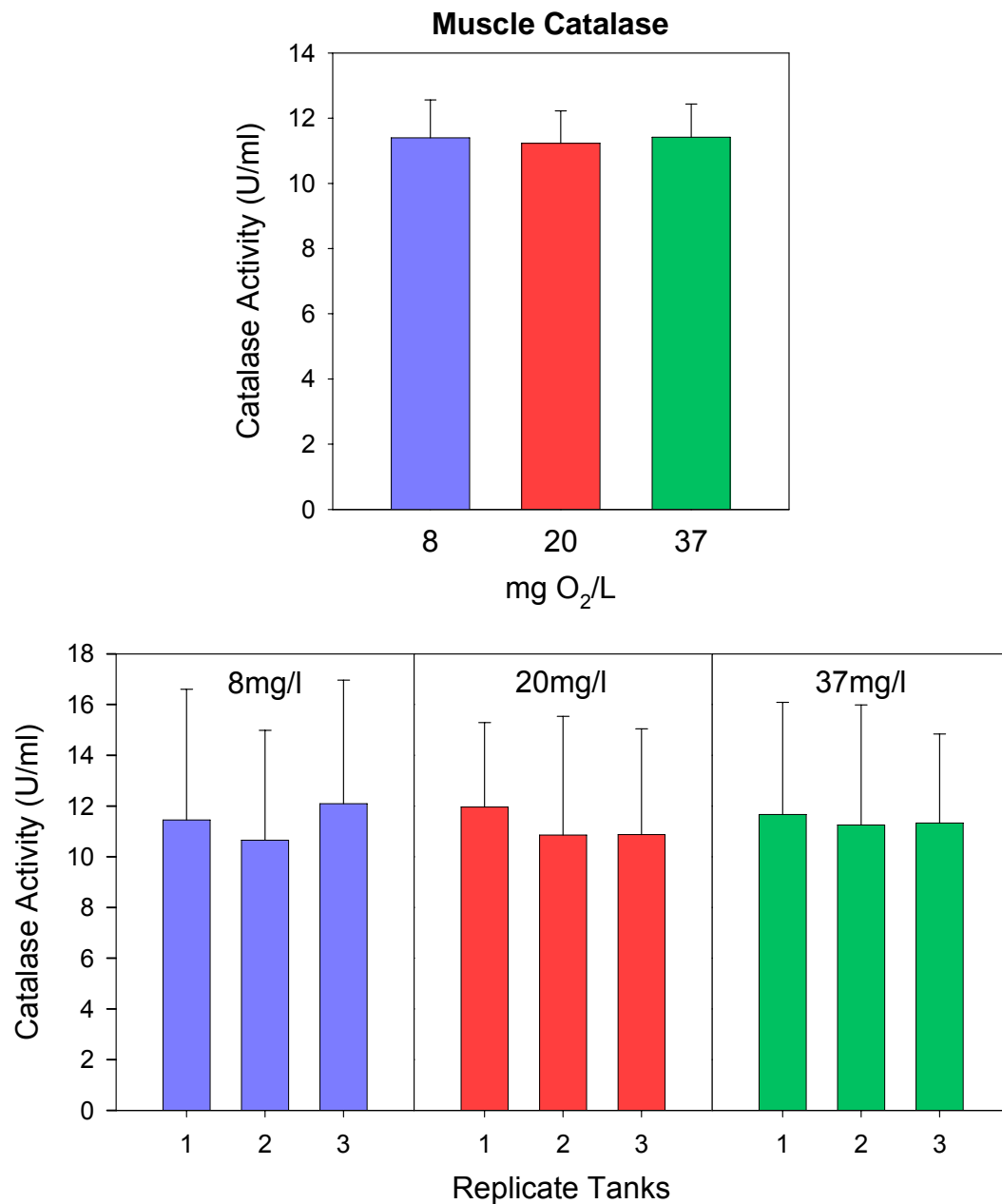


Figure 20d. Catalase activity in Muscle tissue of fish from the complete laboratory experiment. There were no significant differences between replicates within each DO level ($P=0.997$, mean \pm s.d.) or between DO levels overall ($P=0.992$, mean \pm s.e., $n=15$) as determined by a one-way nested ANOVA.

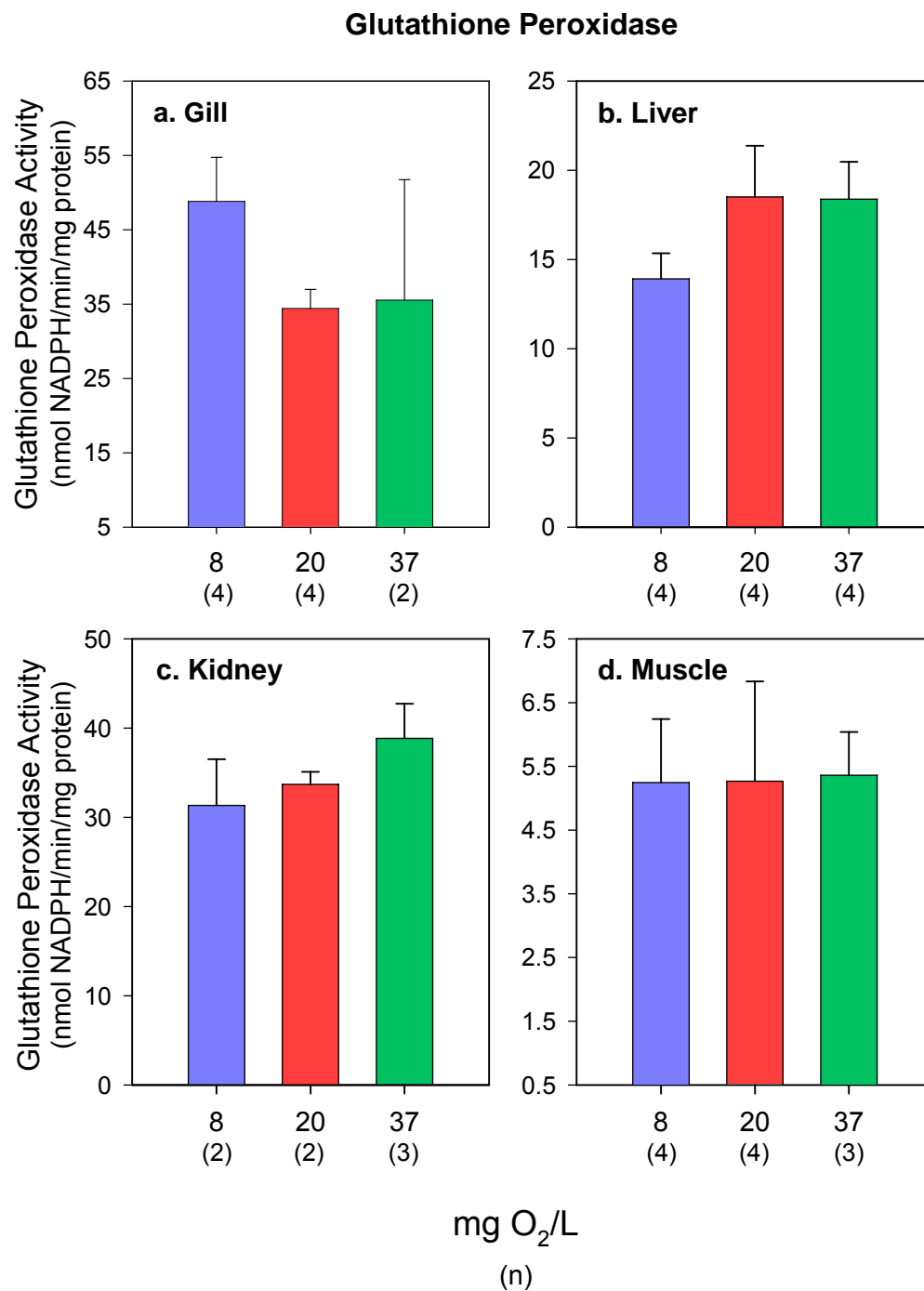


Figure 21. Glutathione Peroxidase activity in a. Gill, b. Liver, c. Kidney, d. Muscle tissue from fish in the preliminary experiment (mean \pm s.e., (n)). There were no significant differences between DO levels in gill ($P=0.265$), liver ($P=0.297$), kidney ($P=0.444$) or muscle ($P=0.998$) based on a one-way ANOVA.

Stockton DWSC O₂ and Temperature

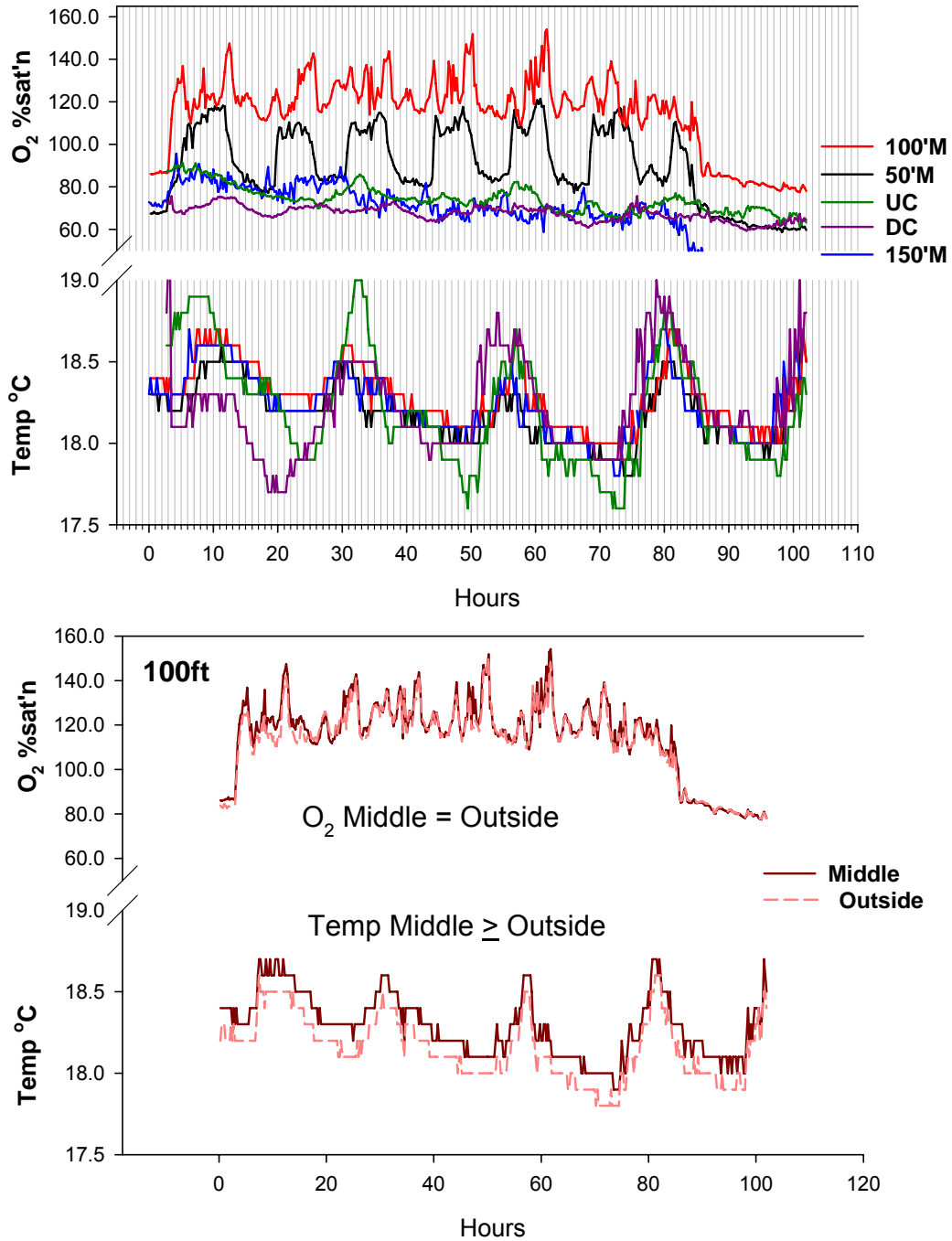
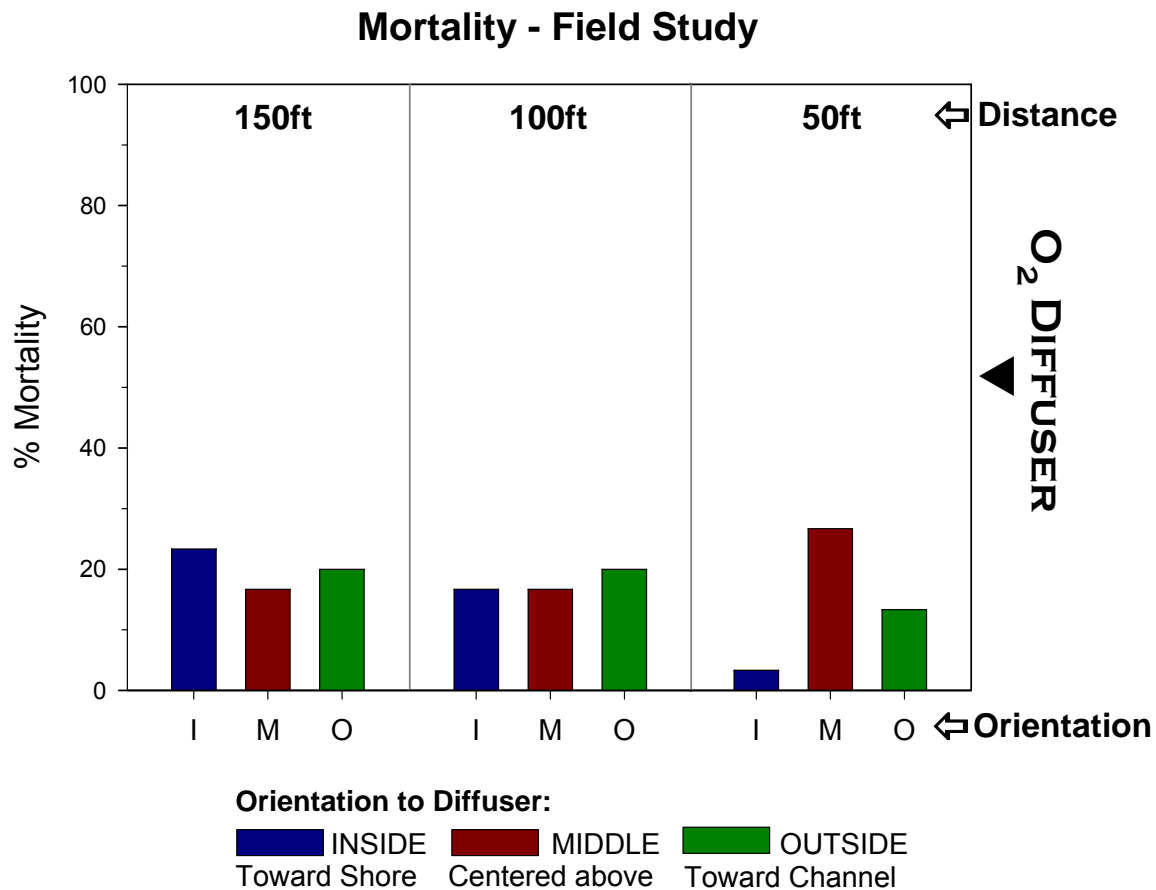


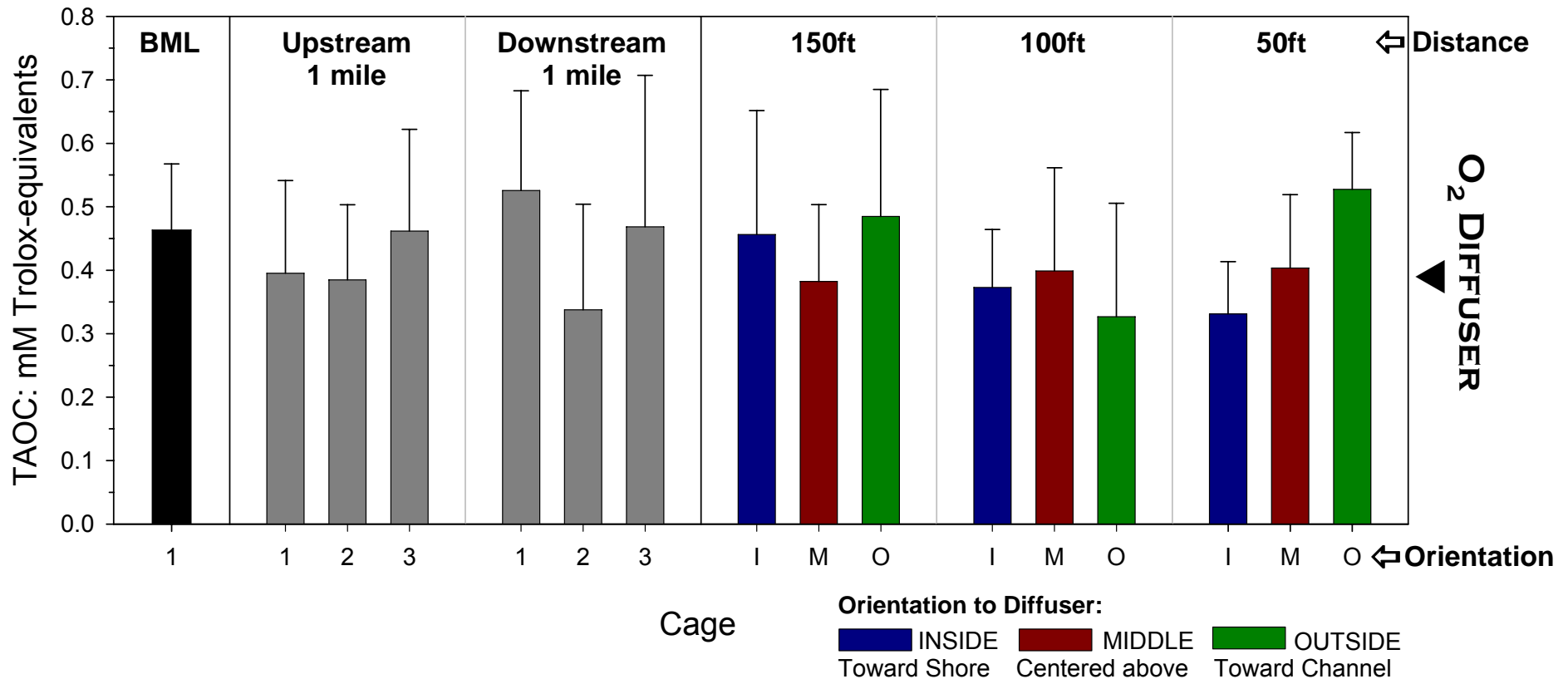
Figure 22. Temperature and DO levels during operation of the oxygen diffuser in the DWSC during the 5-day field study. BML Eureka probes were placed in upstream (UC) and downstream (DC) control cages and in middle diffuser cages at 50 ft, 100 ft, and 150 ft from the diffuser origin (upper graph) as well as in the outside cage at 100 ft.



ANOVA Table					
Source of Variation	DF	SS	MS	F	P
Distance	4	274.073	68.518	1.555	0.276
Orientation	2	54.815	27.407	0.622	0.561
Residual	8	352.593	44.074		
Total	14	681.481	48.677		

Figure 23. Mortality within the diffuser cages (n=30 for each cage). A two-way ANOVA revealed no significant differences due either to distance from the diffuser (50, 100, 150ft) or orientation to it (Inside, Middle, Outside).

Plasma TAOC - Field Study



2-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Distance	2	0.0741	0.0371	1.617	0.208
Orientation	2	0.0295	0.0147	0.643	0.530
D x O	4	0.121	0.0303	1.322	0.273
Residual	56	1.283	0.0229		
Total	64	1.478	0.0231		

1-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Between Groups	15	0.399	0.0266	1.089	0.376
Residual	102	2.491	0.0244		
Total	117	2.890			

Figure 24. Plasma TAOC in juvenile salmon from the field study (mean \pm s.d.). There were no significant differences due either to distance from diffuser or orientation to it as determined by a two-way ANOVA. There were also no differences between any of the diffuser cages and any of the control cages (one-way ANOVA, $P=0.376$). See figs. 3,4, and 5 for position of experimental and control cages in the DWSC.

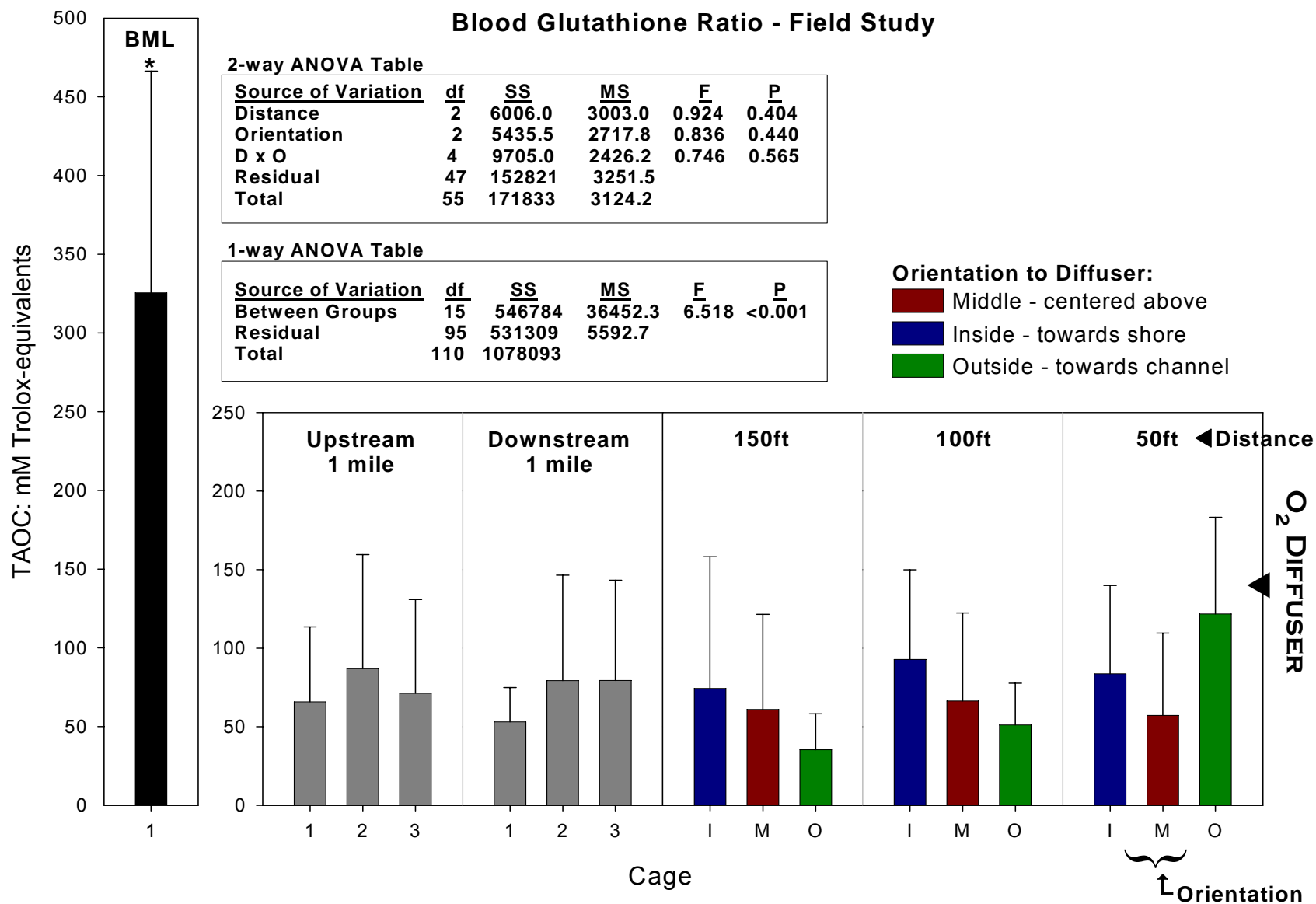
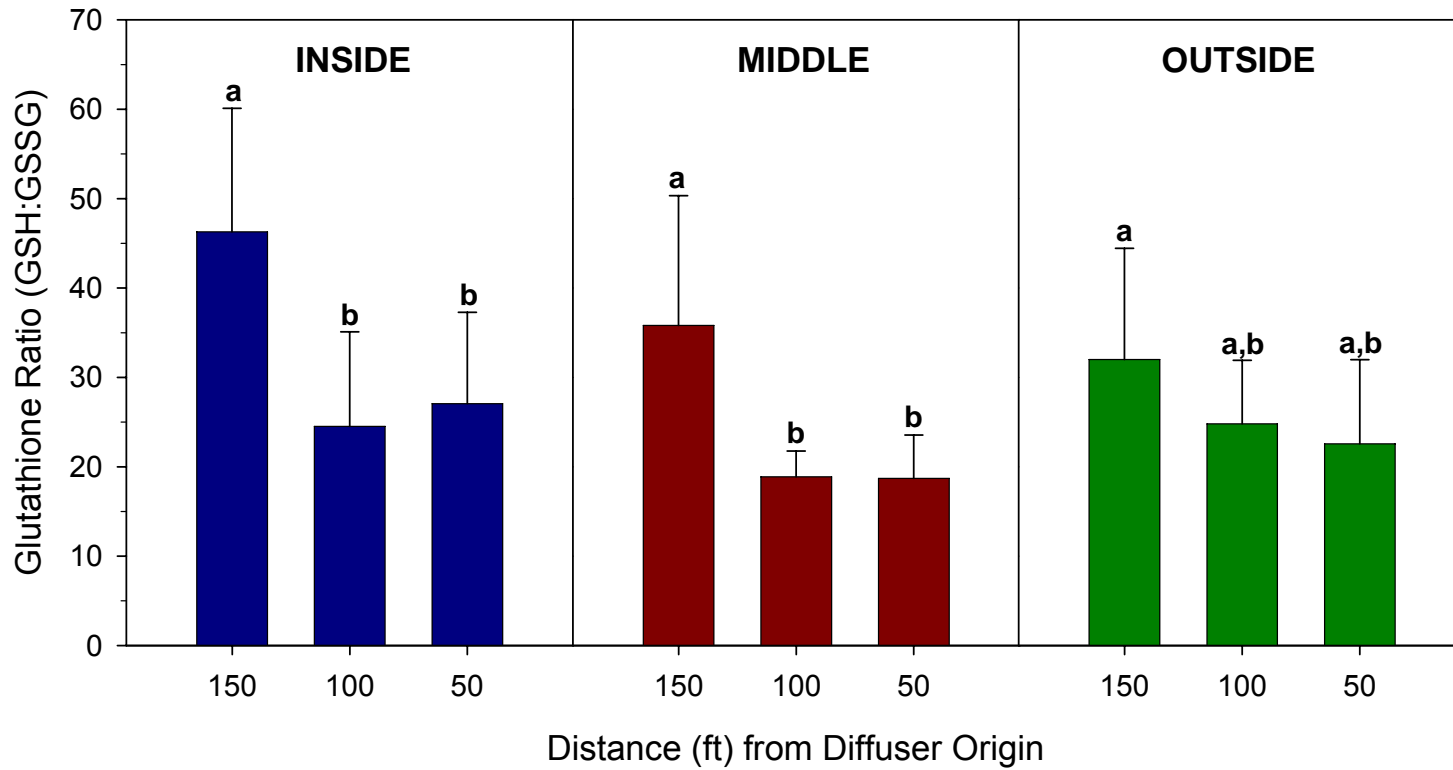


Figure 25. Glutathione ratios in blood of juvenile salmon from the field study (mean \pm s.d.). There were no significant differences due either to distance from diffuser or orientation to it as determined by a two-way ANOVA. There were also no significant differences between any of the river cages (diffuser or control), however the laboratory control (BML) was significantly greater than each of the river cages (one-way ANOVA + SNK testing, $P < 0.05$). See figs. 3,4, and 5 for position of experimental and control cages in the DWSC.

Kidney Glutathione Ratio - Field Study

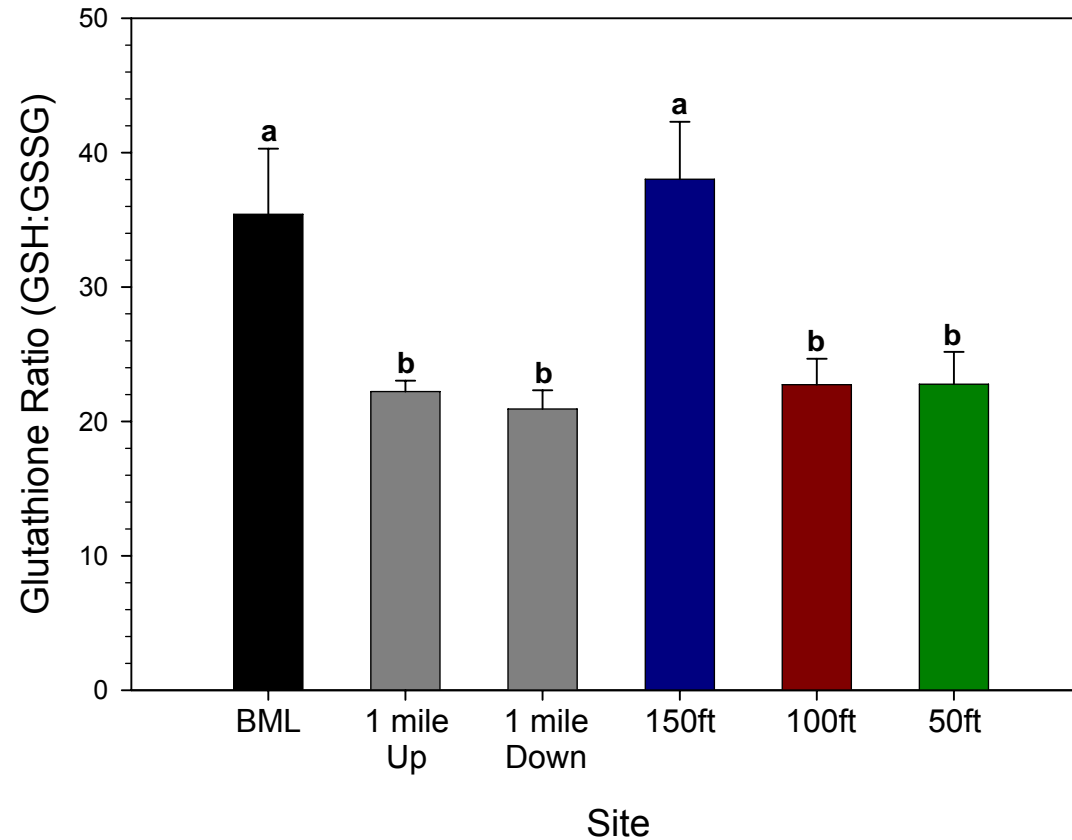


2-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Distance	2	4280.4	2140.2	20.261	<0.001
Orientation	2	1060.1	530.07	5.018	0.009
D x O	4	548.10	137.02	1.322	0.273
Residual	74	7816.7	105.63		
Total	82	13955.2	170.18		

Figure 26a. Glutathione ratios in kidney tissue from juvenile salmon after 5 days in the San Joaquin River at the Stockton DWSC (mean \pm s.d.). A two-way ANOVA was significant for both Distance (150>100, 50ft, $P<0.001$) and Orientation (I>M,O, $P=0.009$). Aposteriori testing within Distance groups (one-way ANOVA + Tukey) revealed that overall 50 and 100ft cage ratios were significantly less than 150ft cage ratios, and more specifically 50ft, 100ft < 150ft for both Inside and Middle orientations but not for Outside cages. Aposteriori testing within Orientation groups revealed that overall Middle cage ratios were less than Inside cage ratios; however more specific testing did not reveal any significant difference between orientations within a given distance (see text for P values).

Kidney Glutathione Ratio - Field Study

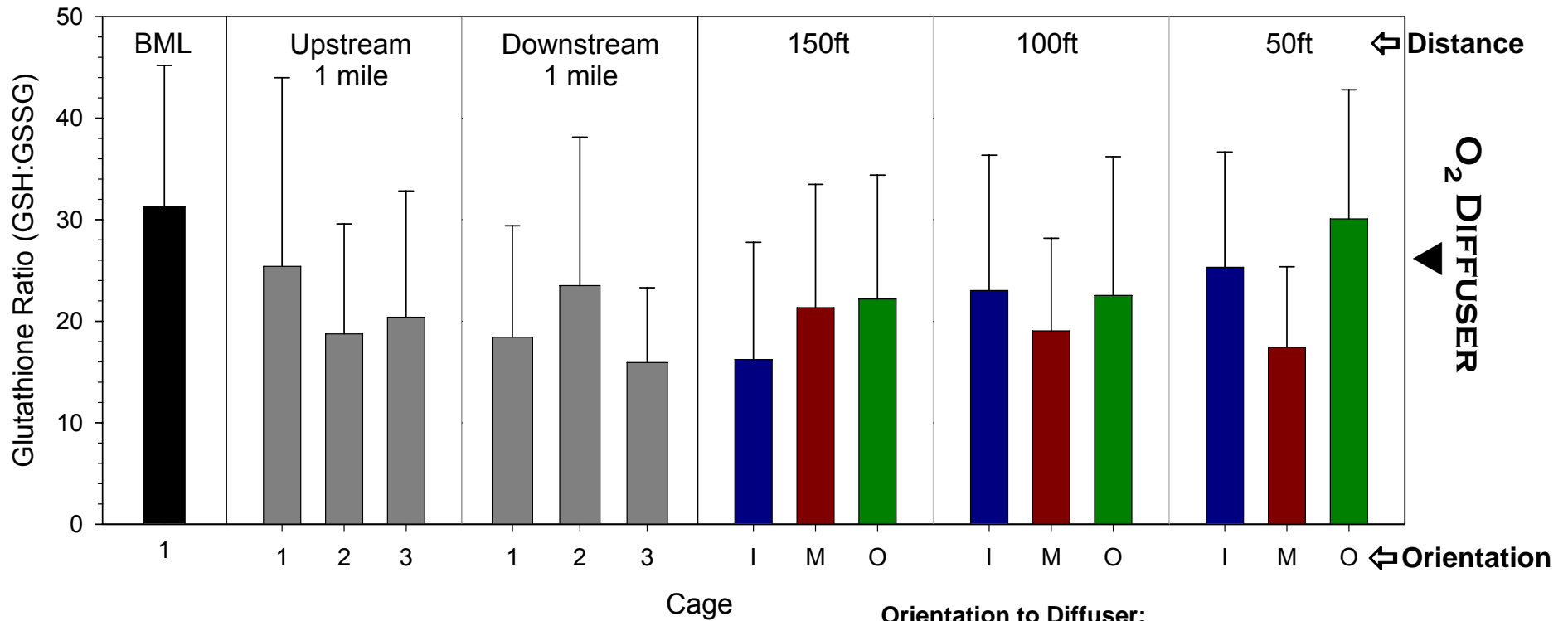


1-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Between Groups	15	6857.3	1371.5	12.08	<0.001
Residual	146	16579	113.56		
Total	151	23437			

Figure 26b. Kidney glutathione ratios from the field study: control vs. experimental cages. Data was combined within distance for experimental cages and analyzed with field and laboratory controls using a one-way ANOVA which was significant ($P < 0.001$). Aposteriori testing revealed the following differences: 100ft, 50ft, and Upstream and Downstream controls were all significantly less than BML controls and 150ft.

Liver Glutathione Ratio - Field Study



2-way ANOVA Table

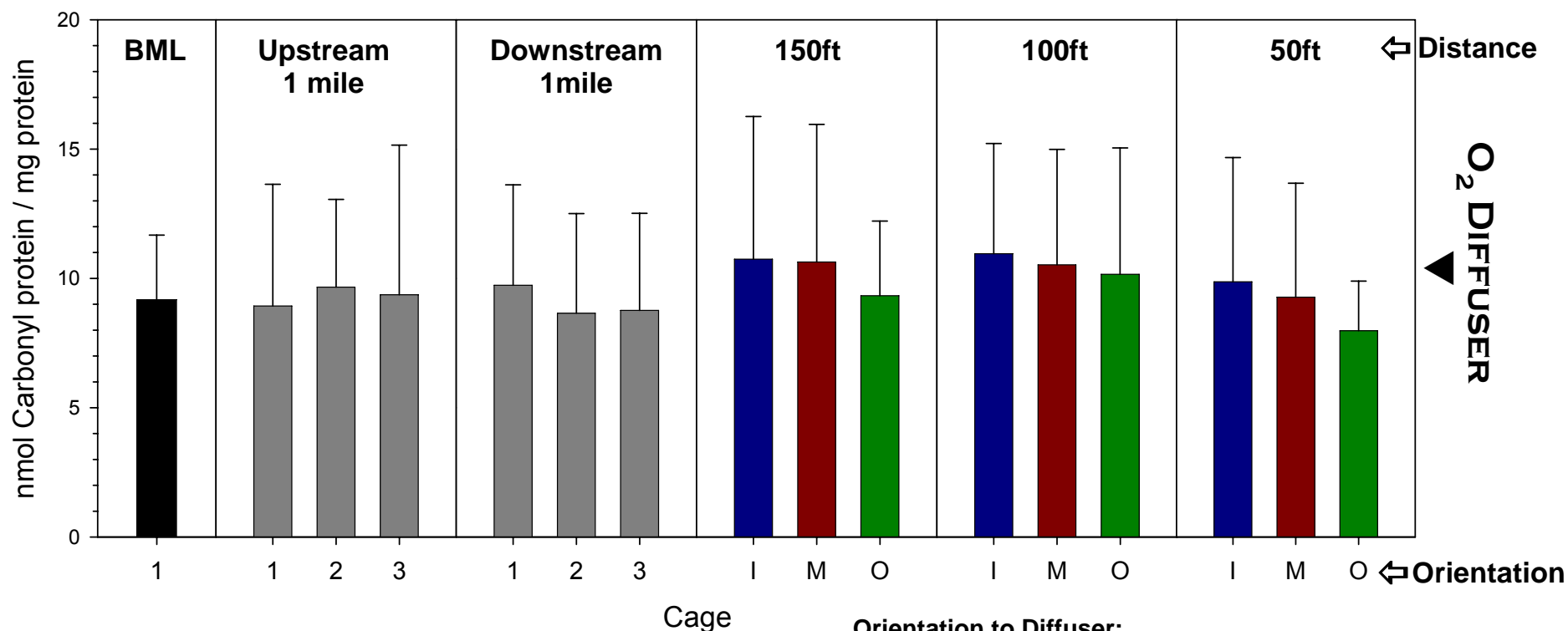
Source of Variation	df	SS	MS	F	P
Distance	2	177.26	88.628	0.655	0.523
Orientation	2	309.56	154.78	1.143	0.325
D x O	4	425.19	106.30	0.785	0.539
Residual	61	8257.5	135.37		
Total	69	9027.1	130.83		

Orientation to Diffuser:

■ INSIDE Toward Shore
 ■ MIDDLE Centered above
 ■ OUTSIDE Toward Channel

Figure 27. Liver glutathione ratios in juvenile salmon from the field study (mean \pm s.d.). There were no significant differences between cages due either to Distance from diffuser or Orientation to it as revealed by a two-way ANOVA. There were no significant differences between any of the cages based on a Kruskal-Wallis one-way ANOVA on Ranks, $P=0.621$ (run due to non-normal distribution; no ANOVA table generated).

Liver Carbonyl Protein - Field Study



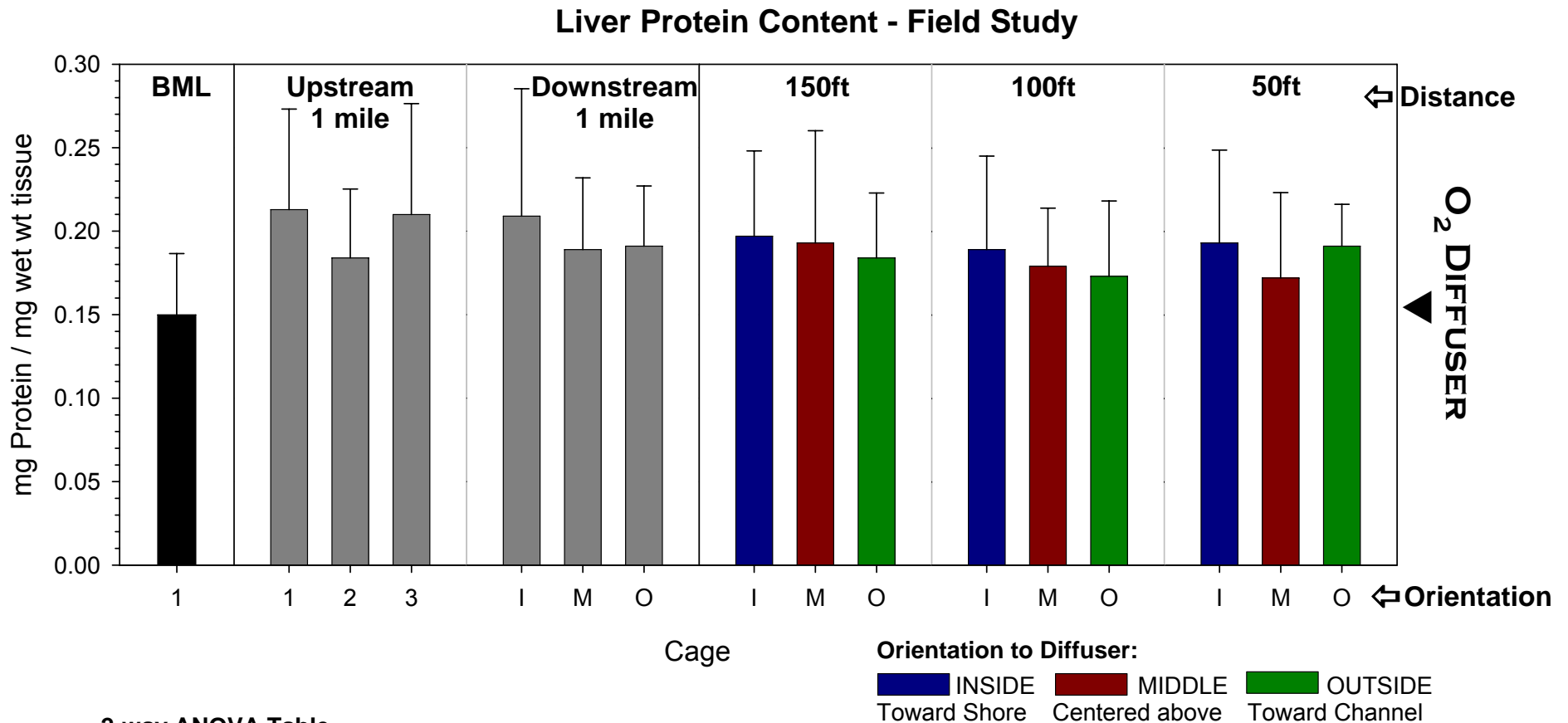
2-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Distance	2	24.910	12.455	0.607	0.549
Orientation	2	19.364	9.682	0.472	0.626
D x O	4	3.022	0.756	0.0368	0.997
Residual	56	1149.8	20.532		
Total	64	1189.7	18.588		

Orientation to Diffuser:

■ **INSIDE** (Toward Shore)
 ■ **MIDDLE** (Centered above)
 ■ **OUTSIDE** (Toward Channel)

Figure 28a. Liver Carbonyl protein content in juvenile salmon from the field study (mean \pm s.d.). There were no significant differences between cages due either to Distance from diffuser or Orientation to it as revealed by a two-way ANOVA. There were no significant differences between any of the cages based on a Kruskal-Wallis one-way ANOVA on Ranks, $P=0.996$ (run due to non-normal distribution; no ANOVA table generated).



2-way ANOVA Table

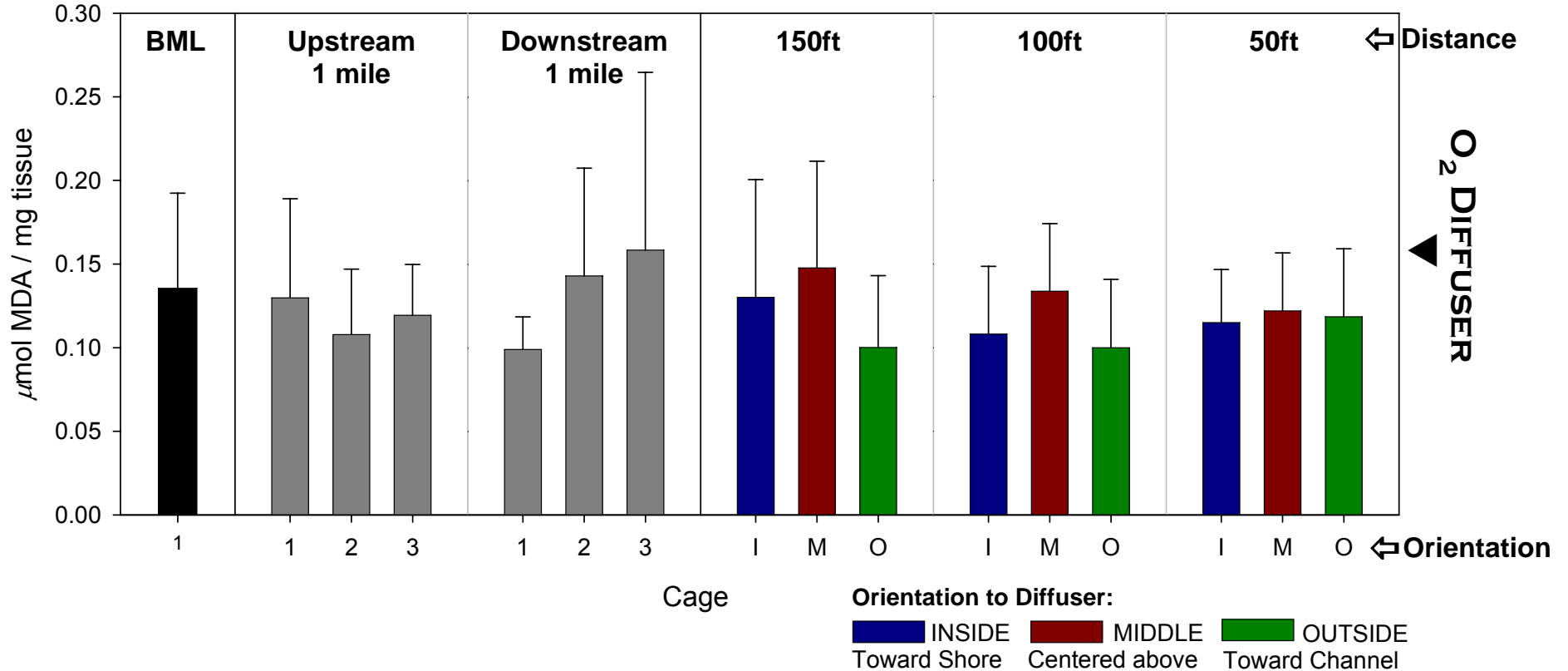
Source of Variation	df	SS	MS	F	P
Distance	2	1.26x10 ⁻³	6.28x10 ⁻⁴	0.253	0.777
Orientation	2	1.83x10 ⁻³	9.14x10 ⁻⁴	0.368	0.694
D x O	4	1.50x10 ⁻³	3.76x10 ⁻⁴	0.151	0.962
Residual	56	0.139	2.48x10 ⁻³		
Total	64	0.144	2.25x10 ⁻³		

1-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Between Groups	15	0.0316	0.00211	0.786	0.690
Residual	107	0.287	0.00268		
Total	122	0.319			

Figure 28b. Liver total protein content in juvenile salmon from the field study (mean \pm s.d.). There were no significant differences between cages due either to Distance from diffuser or Orientation to it as revealed by a two-way ANOVA. There were no significant differences between any of the cages based on a one-way ANOVA P=0.690.

Liver TBARS (Thiobarbituric Acid Substances) - Field Study



2-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Distance	2	0.0016	0.0008	0.371	0.692
Orientation	2	0.0079	0.0040	1.836	0.169
D x O	4	0.0034	0.0008	0.397	0.810
Residual	55	0.119	0.0022		
Total	63	0.134	0.0021		

1-way ANOVA Table

Source of Variation	df	SS	MS	F	P
Between Groups	15	0.0374	0.00249	0.868	0.602
Residual	106	0.304	0.00287		
Total	121	0.342			

Figure 29. Liver TBARS in juvenile salmon from the field study (mean \pm s.d.). There were no significant differences between cages due either to Distance from diffuser or Orientation to it as revealed by a two-way ANOVA. There were no significant differences between any of the cages based on a one-way ANOVA, $P=0.602$.

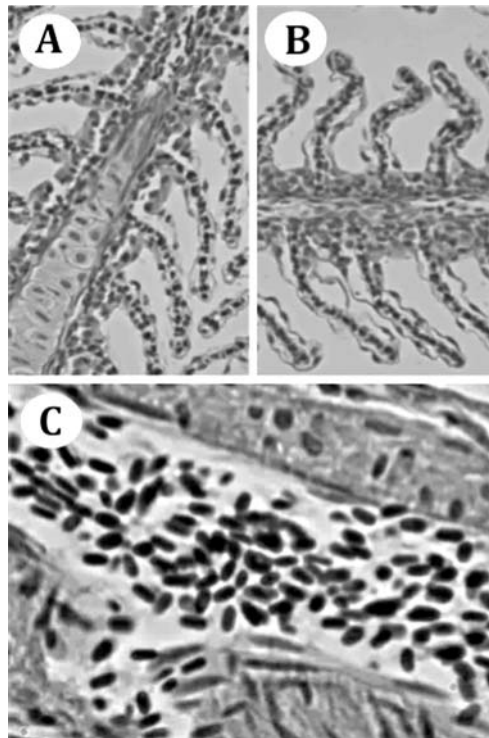


Figure 30. Histology of Chinook salmon gill tissue. A. Gill filament of a control fish. B. Gill filament of a fish treated with 37mg/L DO for five days. C. Gill arch from a fish that was treated with 37mg/L DO for five days. No differences were found between control and hyperoxic fish.

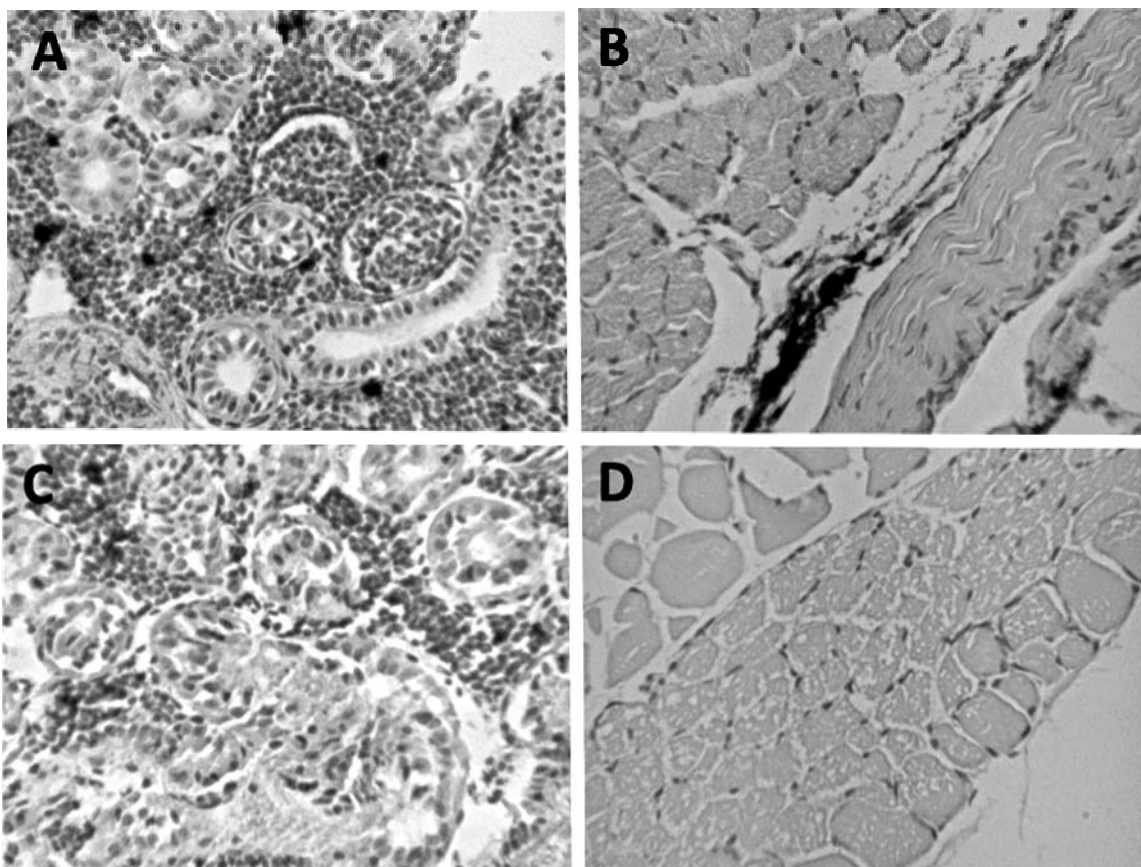
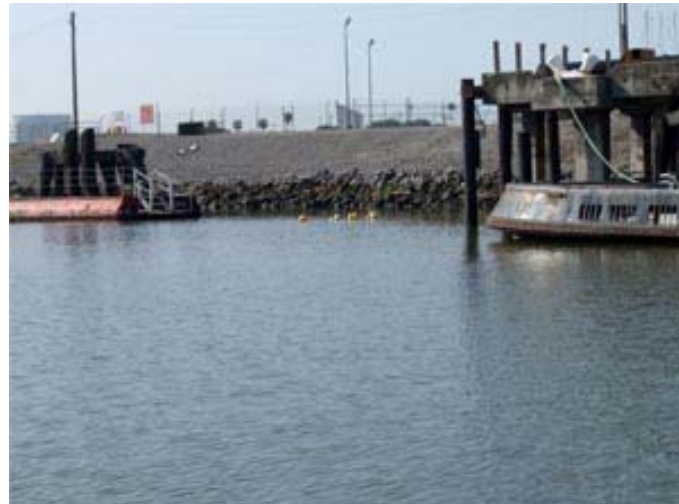


Figure 31. Histological sections of pre-smolt Chinook salmon kidney and skeletal muscle. A & B. Control sections of kidney (A) and skeletal muscle (B) from fish exposed to ambient DO (8mg/L). C & D. Sections of kidney (C) and skeletal muscle (D) from fish exposed to 37mg/L DO. No differences were found between control and hyperoxic fish.

APPENDICES

APPENDIX I. Photographs of cage deployment and retrieval in field study.

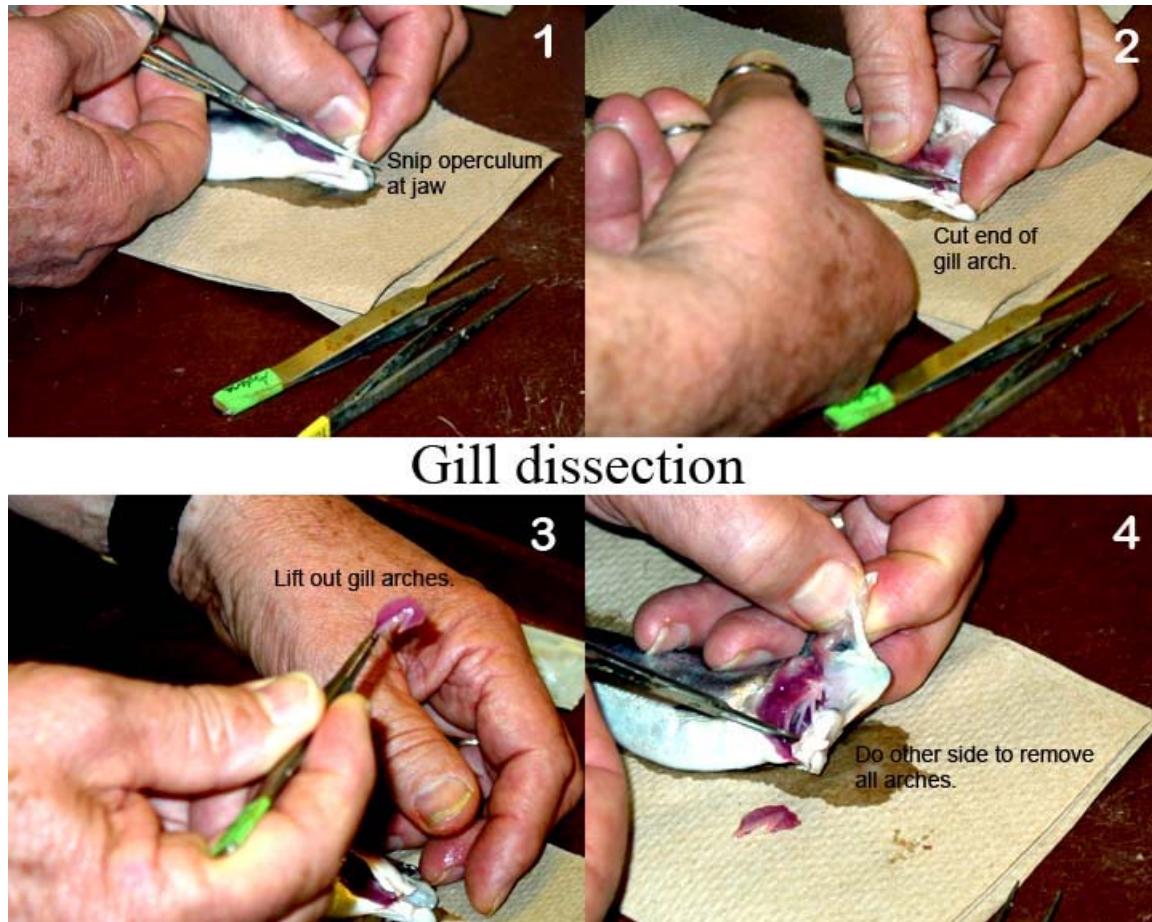


Experimental cage rafts were placed under the dock and over the diffuser. Yellow floats indicate positions of inner, middle and outer cages in the rack (top photo). The upstream control cage rack was positioned south of the dock (bottom photo).



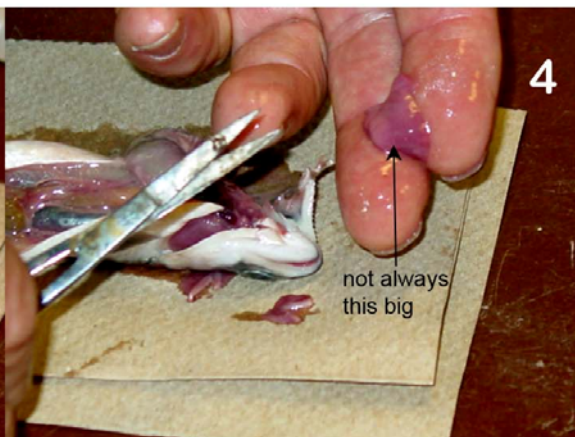
Fish were transported to and from cage racks by boat in 5 gallon lidded buckets (top photo). Cage racks were raised, fish were transferred to and from cages, and cages were lowered back into the DWSC (lower photo).

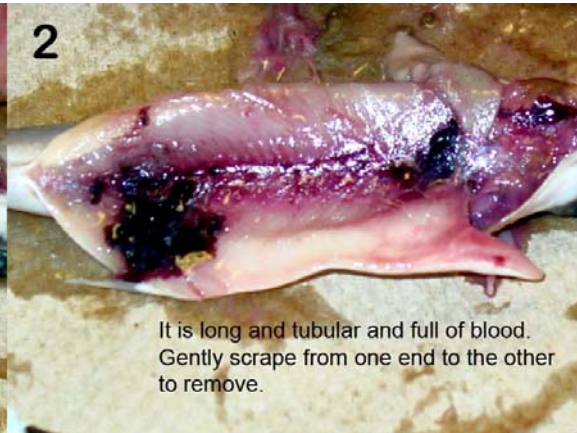
APPENDIX II. Tissue dissection for histology and oxidative stress assays.





Liver Dissection





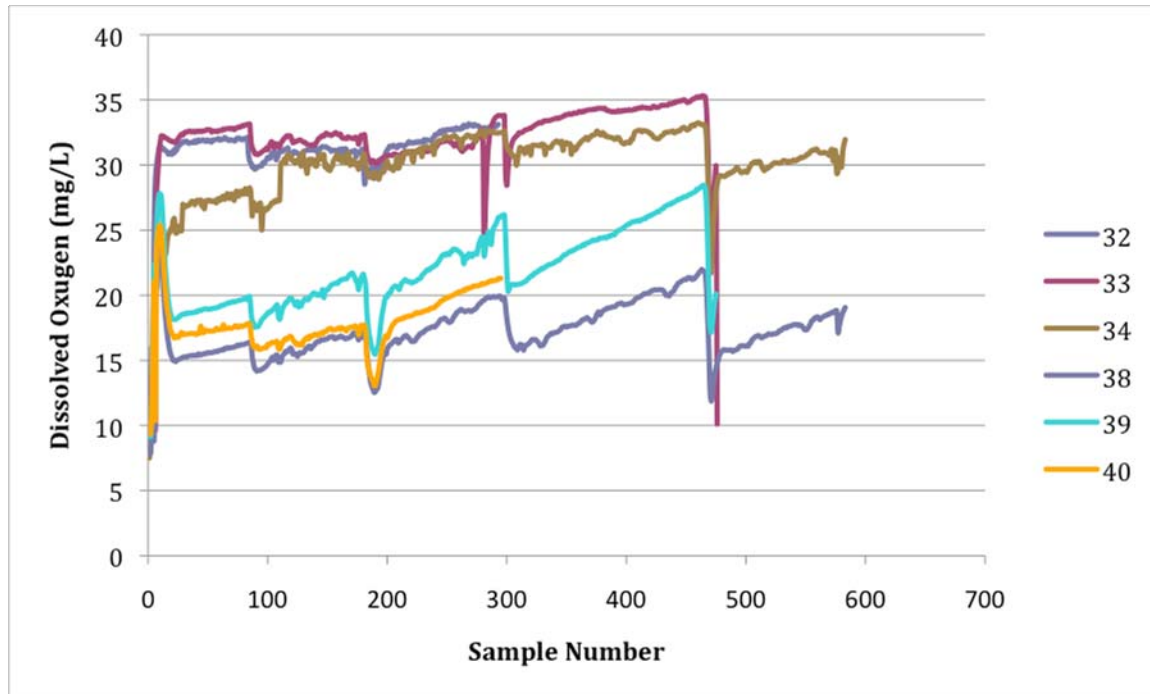
Kidney (above)

Muscle (below)



APPENDIX III: DO readings for preliminary experiment 3.

Data is on attached CD File = APPENDIX III Prelim Exp 3 DO DATA 12-5.xls



Graph is of raw DO data (12-4 MIDGE O2-DATA sheet) from six Midge DO sensors. Sensors 32, 33, and 34 were in 400% targeted DO water and sensors 38, 39, and 40 were in 200% targeted DO water. Readings were collected at 15 minute intervals 12/4 – 12/10 07.

APPENDIX IV:**DO DATA FOR FIGs 7 & 8, COMPLETE EXPERIMENT****CD File = APPENDIX IV COMPLETE EXP DO DATA 12-13.xls****FIGURE 7: POLARIS am O2 DATA & GRAPH FOR 12-13 LAB EXP**

Date		32	33	34	35	36	37	38	39	40
		O2 mg/L	O2 mg/L	O2 mg/L	O2 mg/L	O2 mg/L	O2 mg/L	O2 mg/L	O2 mg/L	O2 mg/L
12/14	am	36.5	38.3	38.8	7.6	7.7	7.9	20.7	20.8	20.6
12/15	am	37.2	39.9	38.7	8.7	8.9	8.7	25.5	25.6	24.1
12/16	am	36.9	37	37.5	8.1	8.3	8.5	21.4	21.6	20.5
12/17	am	35.1	36.4	36.6	8.7	8.4	8.3	18.3	19.5	18.9
12/18	am	37.6	39.2	41.6	8.9	8.2	8.6	23	25.7	24.8
12/19	am	35.2	37.6	32.7	8.3	7.9	8.3	20.7	21.1	19.5

SEAWATER CHALLENGE BEGUN ON 12/19**FIGURE 8: AVERAGE MIDGE & POLARIS DATA PER DAY****MIDGE READINGS**

Date	32 O2 mg/L	33 O2 mg/L	34 O2 mg/L	AVE	STD DEV	38 O2 mg/L	39 O2 mg/L	40 O2 mg/L	AVE	STD DEV
12/14	31.75	29.14	25.22	28.70	3.29	13.30	18.40	16.29	16.00	2.56
12/15	31.49	28.63	24.72	28.28	3.40	12.67	17.52	15.38	15.19	2.43
12/16	31.70	28.85	24.06	28.20	3.86	12.28	16.84	13.85	14.32	2.32
12/17	31.88	28.88	23.77	28.18	4.10	12.97	17.94	14.62	15.18	2.53
12/18	31.84	28.72	23.68	28.08	4.12	13.72	19.13	15.81	16.22	2.73
12/19	33.09	29.62	24.23	28.98	4.46	13.78	18.87	15.62	16.09	2.58
AVE	31.96	28.97	24.28			13.12	18.12	15.26		
STD DEV	0.57	0.36	0.59			0.59	0.86	0.88		

POLARIS READINGS

Date	32 O2 mg/L	33 O2 mg/L	34 O2 mg/L	AVE	STD DEV	38 O2 mg/L	39 O2 mg/L	40 O2 mg/L	AVE	STD DEV
12/14	36.50	38.30	38.80	37.87	1.21	20.70	20.80	20.60	20.70	0.10
12/15	37.20	39.90	38.70	38.60	1.35	25.50	25.60	24.10	25.07	0.84
12/16	36.90	37.00	37.50	37.13	0.32	21.40	21.60	20.50	21.17	0.59
12/17	35.10	36.40	36.60	36.03	0.81	18.30	19.50	18.90	18.90	0.60
12/18	37.60	39.20	41.60	39.47	2.01	23.00	25.70	24.80	24.50	1.37
12/19	35.20	37.60	32.70	35.17	2.45	20.70	21.10	19.50	20.43	0.83

APPENDIX V

Data for Figure 22, DO probe readings during DWSC field experiment are contained in CD File = APPENDIX V Field DWSC DO DATA.xls

APPENDIX VI

DO Data supplied by the California Department of Water Resources, University of California, Davis, Department of Environmental Sciences, and University of the Pacific are contained in CD File = APPENDIX VI DWR Supplied DES & UOP Data & Plots.xls.

APPENDIX VII

Data from Midge probes collected during DWSC saltwater challenge after DWSC hyperoxia exposure is contained in CD File = APPENDIX VII Field Salt Challenge.xls.