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Determine the Influence of Time Held in "Knockdown" Anesthesia on Survival and Stress of Surgically Implanted Juvenile Salmonids

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January 2012

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Pacific Northwest National Laboratory
Sequim, Washington 99352

Summary

This report summarizes the responses of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) subjected to the anesthetic, tricaine methanesulfonate (MS-222), during surgical implantation of acoustic transmitters (ATs) and passive integrated transponders (PITs). The Columbia Basin Surgical Protocol Steering Committee expressed concern that the recommendation of a 10-minute maximum exposure to MS-222 could be too long for a juvenile salmonid resulting in increased behavioral and physiological costs, and/or decreased survival. These undesirable results are further problematic because survival study assumptions require that fish are representative of fish in the river that have not been subjected to handling, holding, and surgical implantation. Thus, there is a need to determine the amount of time fish can be held in knockdown anesthetic without negatively influencing their survival and thus fish passage survival models. The study reported here examined the relationship between stress indicators for several blood analytes and biochemistries (sodium, calcium, potassium, blood pH, plasma cortisol), time under anesthesia, and number of days post-exposure to anesthesia in juvenile yearling Chinook salmon (YCH). Fish were randomly assigned to one of three treatment groups:

- anesthetic control (no anesthetic exposure, no surgical implantation)
- surgical control (exposed to anesthesia and removed when reached Stage 4 anesthesia, no surgical implantation)
- one of seven treatments where fish remained in the anesthetic for 0, 3, 5, 6, 9, 12, or 15 minutes beyond the "knockdown" time (KD) when they reached Stage 4 anesthesia and were surgically implanted with an AT and PIT.

Controls and KD treatment exposure groups were further assigned to one of four post-surgical implantation-monitoring days: 0, 1, 7, or 14 days after treatment. Day 0 fish were immediately sampled after the anesthetic treatment; while 1, 7, and 14 day fish were held until their respective day for sampling and termination. The purpose of the study was to address three questions:

1. Did the MS-222 KD treatment exposure groups result in differential mortality for surgically implanted fish?
2. Did the stress responses of juvenile Chinook salmon vary significantly as a result of KD on day 0 and when compared to the anesthetic control fish on day 0?
3. Did the stress responses of juvenile Chinook salmon vary significantly as a result of the KD when examined on post-surgical implantation-monitoring days 1, 7, 14; and when compared to surgical control fish over the same time periods?

Although we expected to see mortalities in the 12- and 15-minute extended KD treatment exposures, there were no mortalities in any of the controls or KD exposure times. This suggests that perhaps: 1) the duration of exposure (KD), at 80 mg/L, in anesthetic prior to surgical implantation does not directly affect fish survival over the time range tested; 2) the life stage of holdover juvenile YCH may be less susceptible to anesthetic stress and thus mortality than actively outmigrating juvenile YCH; or 3) juvenile YCH that are actively outmigrating, when subjected to repeated exposure to MS-222, are more likely to experience higher rates of mortalities as seen in field research. Blood analytes (sodium, potassium, and calcium) are not strong indicators of stress, but are more likely indicators of secondary stress responses and/or osmatic imbalance. Blood analyte levels of the day 0 post-implantation 6- and 9-minute

knockdown treatments were greater than the 0-, 3-, 5-, 12-, and 15-minute knockdown treatment levels on day 0. Yearling Chinook salmon in the 6- and 9-minute knockdown treatments tended to have blood analyte values that decreased over post-implantation monitoring days; yet were consistently different from the other knockdown time treatments on each post-surgical implantation day. There were no knockdown time treatments that resulted in overall reduced blood biochemistry measures, but a general pattern of reduced measures could be detected over the post-implantation monitoring days. This indicated that anesthetic and surgical stresses experienced on day 0, and subsequent changes in physiological state were generally reduced by day 14.

Overall, this study did not reveal any consistent trends among the juvenile Chinook salmon responses that indicated one treatment time interval was more beneficial (e.g., overall lower stress response) than the other time treatment intervals. The knockdown time treatments of 6 and 9 minutes yielded results different from other time treatments regardless of the post-implantation monitoring day. However, the physiological state changes or the progression of changes at 6 and 9 minutes compare to the other time points remains unclear. It was apparent that surgically implanted YCH exhibited an overall higher stress response, as indicated by the biochemistry data, than those not surgically implanted with ATs and PITs, suggesting that surgery itself may mask the effects of the anesthetics. Based on this, we recommend that the "10-minute maximum exposure to MS-222" suggested by the committee remain in place, until further research indicates anesthetic time points or surgical procedures that result in any measureable mitigation or reduction in the overall stress response of juvenile YCH during the surgical implantation process. Other factors not evaluated in this report, that initiate or can alter stress responses, include anesthetic dose, water hardness, temperature, fish size, and past exposure history.

Acknowledgments

This project was funded by the U.S. Army Corps of Engineers (USACE), Portland, Oregon. We thank M. Brad Eppard, the USACE contracting officer's representative, for his oversight and guidance; the Columbia Basin Surgical Protocol Steering Committee (CBSPSC) for its active discussion and hypotheses on the effects of tricaine methanesulfonate on outmigrating salmon held in Stage 4 anesthesia for various periods of time. We also thank the following Pacific Northwest National Laboratory staff and interns for their assistance with this research: Carmina Arimescu, Jim Boyd, Scott Carpenter, Jessica Carter, Kathleen Carter, Alison Colotelo, Joanne Duncan, Marybeth Gay, Mathew Hennen, Benjamin Miller, Eric Oldenburg, Ida Royer, Andy Solcz, and Mark Weiland. The statistics were conducted by Adam Seaburg and John Skalski, University of Washington. We greatly appreciate the use of PNNL laboratory space and equipment from Ann Miracle and Amoret Bunn. Lastly, we would like to thank our thorough reviewer and editor, Michael Cobb and Susan Ennor, for their detailed editing and excellent comments.

Animal facilities were certified by the Association for Assessment and Accreditation of Laboratory Animal Care; animals were handled in accordance with federal guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee, Battelle–Pacific Northwest Division. Trade names references do not imply endorsement by the U.S. Government.

Acronyms and Abbreviations

°C	degree(s) Celsius (or Centigrade)
AC	fish randomly assigned as anesthetic controls (no anesthetic exposure, no surgical implantation
ANODEV	Analysis of Deviance
AT	acoustic transmitter
CBSPSC	Columbia Basin Surgical Protocol Steering Committee
Ca^{2+}	calcium
Df	degree of freedom
EIA	enzyme immunoassay
F	F-test statistics
FCRPS	Federal Columbia River Power System
FL	fork length
$\mu g\ L^{-1}$	microgram(s) per liter
g	gram(s)
g	relative centrifugal force
JSATS	Juvenile Salmon Acoustic Telemetry System
K^+	potassium
KD	fish randomly assigned to an anesthetic "knockdown" time treatment (time treatment fish were exposed to anesthetic after the induction of stage 4 anesthesia)
kg/m^3	kilogram(s) per cubic meter
L	liter(s)
g	gram(s)
m^3	cubic meter(s)
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
MS-222	tricaine methanesulfonate
Na^+	sodium
nm	wavelength
P	p-value; probability of test statistics
pH	pH
PIT	passive integrated transponder
PNNL	Pacific Northwest National Laboratory

SC	fish randomly assigned as surgical controls (exposed to anesthesia at time of surgery, no surgical implantation)
SD	standard deviation
WW	wet weight
YCH	yearling Chinook salmon

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1.0 Introduction

The Juvenile Salmon Acoustic Telemetry System (JSATS) was developed for the U.S. Army Corp of Engineers Portland District (USACE) to address questions related to survival and performance measures of juvenile salmonids as they pass through the Federal Columbia River Power System (FCRPS). Researchers using JSATS acoustic transmitters (ATs) were tasked with standardizing the surgical implantation procedure to ensure that the stressors of handling and surgery on salmonids were consistent and less likely to cause effects of tagging in survival studies. Researchers questioned whether the exposure time in "knockdown" anesthesia (or induction) to prepare fish for surgery could influence the survival of study fish (CBSPSC 2011). Currently, fish are held in knockdown anesthesia after they reach Stage 4 anesthesia until the completion of the surgical implantation of a transmitter, which ranges from 5 to 15 minutes for studies conducted in the Columbia Basin. The Columbia Basin Surgical Protocol Steering Committee (CBSPSC[1]) expressed concern that its currently recommended 10-minute maximum time limit for fish to be held in anesthetic —tricaine methanesulfonate (MS-222, 80 mg L^{-1} water) — could increase behavioral and physiological costs, and/or decrease survival of outmigrating juvenile salmonids. In addition, the variability in the time fish are held at Stage 4 could affect the data intended for direct comparison of fish within or among survival studies. Under the current recommended protocol, if fish exceed the 10-minute time limit, they are to be released without surgical implantation, thereby increasing the number of fish handled and endangered species "take" at the bypass systems for FCRPS survival studies.

To address the CBSPSC concerns, researchers at Pacific Northwest National Laboratory (PNNL) conducted the study reported here to determine the anesthetic (MS-222; 80 mg L^{-1}) exposure time yearling Chinook salmon (YCH; *Oncorhynchus tshawytscha*) could be held in Stage 4 anesthesia, without negatively affecting YCH recovery or survival. After the induction of Stage 4 anesthesia, fish were held in knockdown anesthetic for various exposure periods and then surgically implanted with a passive integrated transponder (PIT) and JSATS AT. The time periods fish were held in knockdown after reaching Stage 4 anesthesia were 0, 3, 5, 6, 9, 12, or 15 minutes. The effects of extended exposure to MS-222 after Stage 4 anesthesia were determined by 1) blood analyte concentrations (Na^+, K^+, and Ca^{2+}), 2) blood pH, 3) plasma cortisol, and 4) the survival of exposed fish.

1.1 Background

In aquaculture and fisheries research, anesthesia is commonly used to minimize stress responses and injuries related to events like crowding, handling, netting, transportation, blood sampling, surgeries, and exposure to air (Burka et al. 1997; Ross and Ross 2008; Carter et al. 2011; Zahl et al. 2011). A number of anesthetics have been used and evaluated for fisheries use, but issues associated with drug induction, toxicity to humans and fish, safety for humans, waste/disposal, and cost have reduced effective drug options. MS-222, benzocaine, carbon dioxide, clove oil, and isoeugenol are the most common anesthetics used on fish (Ross and Ross 2008). MS-222, also known as Tricaine-S or Finquel, is classified as a local

[1]CBSPSC is a committee that provides oversight and a minimum standard of practice for how research fish are utilized in the Anadromous Fish Evaluation Program (AFEP). Surgical protocols were based on a thorough review of the scientific literature, current research, group discussions among experts in the field of biotelemetry and fish physiology, and a consensus of the Steering Committee.

ester type anesthetic that is the only anesthetic approved by the U.S. Food and Drug Administration for use with food fish in the United States of America and European Union (Carter et al. 2011).

MS-222 is an isomer of benzocaine with an additional sulfonate radical, making it more soluble, yet acidic, in solution (Carter et al. 2011) and easily absorbable through the gills and skin of fish, where it then enters the bloodstream (Hunn and Allen 1974; Ferreira et al. 1984). It is metabolized by acetylation reactions (Burka et al. 1997), and then excreted by the kidney with urine within 24 hours after exposure. Nevertheless, a 21-day withdrawal time is required for fish marketed for human consumption (Kelsch and Shields 1996; Carter et al. 2011). MS-222 immobilizes fish by depressing their central and peripheral nervous systems (Summerfelt and Smith 1990; Carter et al. 2011). The obstruction of the nerve is facilitated by the high lipid solubility of MS-222, allowing for an easy shift across the cell membrane to bind with sodium channels (Hunn and Allen 1974). More specifically, nerve impulses are blocked by inhibiting voltage-sensitive Na^+ channels, thereby reducing the cumulative inward flux of Na^+ (Neumcke et al. 1981; Butterworth and Strichartz 1990). MS-222 affects the higher-level functions of the central nervous system (CNS), but the full biochemical process and implications of the effects are not fully understood (Ueta et al. 2007). Based on behavioral and physiological observations, Summerfelt and Smith (1990) derived a scale for the effects anesthesia has on the CNS, which is visible in six stages, ranging from light sedation (Stage 1) to asphyxia (Stage 6). Stage 4 anesthesia is characterized by a total loss of equilibrium, slow and regular opercular beats, and loss of spinal reflex (Summerfelt and Smith 1990); and is the recommended anesthetic level for invasive surgical procedures (Summerfelt and Smith 1990).

Unfortunately, MS-222 acts as a stressor during induction and throughout the exposure period. The high lipid solubility leads to rapid absorption across the gills. The depression of the CNS affects gill functioning (e.g., depressed gill beating), resulting in bradycardia— a decrease of oxygen uptake and a decrease of carbon dioxide excretion causing hypoxemia— and concomitantly localized acidosis (Iwama et al. 1989; Houston 1971; Ross and Ross 2008). In addition, MS-222 alters other blood chemistry parameters in fish, including glucose and lactate concentrations, hormone concentrations (e.g., cortisol), protein and amino acid concentrations, blood pH, and electrolyte (e.g., K^+, Na^+, Ca^{2+}) concentrations (Davis and Griffin 2004; Cho and Heath 2000; Small 2003; Congleton 2006). Higher concentrations of MS-222 (100–200 mg L^{-1} water) quickly sedate fish and may be effective in minimizing or preventing changes in blood chemistry (Wedemeyer et al. 1990; Barton 2002; Holloway et al. 2004; Congleton 2006). Interestingly, even when fish are deeply sedated, MS-222 has been shown to cause an increase in plasma cortisol concentrations, likely due to over-handling and thus activation of the hypothalamic-pituitary-interrenal axis that produces cortisol (Barton 2002). Blood plasma constituents, such as K^+, Na^+, Ca^{2+}, have been used as a gauge of anesthetic stress response, osmoregulatory state, and overall health of fish (Cataldi et al. 1998; Davis and Griffin 2004; Carter et al. 2011).

Regardless of the side effects, MS-222 is used widely and effectively because of its stability, predictability, and relatively "known" dosages for desired outcomes when anesthetizing fish (Ferreira et al. 1984; Summerfelt and Smith 1990; Thomas and Robertson 1991). The efficacy and stress associated with MS-222 varies with species, age, life history, body size, and sex (Gilderhus and Marking 1987; Stehly and Gingerich 1999; Tsantilas et al. 2006; Carter et al. 2011) as well as water physical-chemical parameters, such as salinity, hardness, pH, oxygen levels, and water temperature (Mylonas et al. 2005; Zahl et al. 2011; Carter et al. 2011). MS-222 has a large safety margin between the effective, maximum safe and euthanasia concentrations, which is desirable for field research or with novice users. Surgical procedures, such as intracoelomic implantation, often recommend an initial anesthesia induction

phase (knockdown) followed by a maintenance anesthesia phase, so that the fish are sedated as quickly, yet safely as possible using higher concentrations of MS-222. The recommended MS-222 concentration for rainbow trout (*O. mykiss*) is 100 mg L^{-1} water to reach Stage 4 within 2 to 4 minutes (depending on water temperature (Kolanczyk et al. 2003; Carter et al. 2011) and 250 mg L^{-1} water for euthanasia (Wilson et al. 2009; Carter et al. 2011). The CBSPSC (2011) recommended MS-222 doses for surgeries to range from 60 to 80 mg L^{-1}, although no reference was provided to explain the recommended dose. While the MS-222 dosages are documented for some fish species, the effects of time spent in the recommended knockdown dosage past the onset of Stage 4 anesthesia (Summerfelt and Smith 1990) yields unknown outcomes on stress and survival of fish.

Stress, altered behavior, recovery time, and survivability for fish after MS-222 exposure can critically affect the results and conclusions of research and monitoring programs. In the FCRPS, researchers tasked with standardizing JSATS AT surgical implantation procedures have noted that the time fish are held in induction anesthesia, ("knockdown" or surgical anesthesia to prepare them for surgery), could influence their survival (CBSPSC 2011). It is recommended that fish reach Stage 4 anesthesia 2 to 3 minutes after being exposed to knockdown anesthetic (Marking and Meyer 1985; Bell 1987; Iwama and Ackerman 1994). Currently, the amount of time fish are held after they reach Stage 4 anesthesia, until surgical implantation of a transmitter, varies from 5 to 15 minutes among studies and research groups, whether in or outside of the Columbia River basin. For example, Finquel® recommends that a "moderately rapid anesthesia" induction time occurs in less than 2-5 minutes (Argent Chemical Laboratories 2011). When fish are "batch sampled" (e.g., typically 3 to 10 fish administered MS-222 through water bath contact), the knockdown and data collection (i.e., length, weight, tag assignment, and fish condition) may take from 5 to 10 minutes depending on personnel experience, experimental design, or whether complications are encountered during data acquisition. The extended knockdown time may lead to adverse effects on fish survival and an inability to compare results directly within or among survival studies.

There is no solid evidence to guide researchers as to the maximum time YCH should be anaesthetized for surgical implantation of telemetry tags, without affecting their physiology and long-term survival. The CBSPSC (2011) recommended, until research proves otherwise, that juvenile salmonids selected for surgical implantation not be held in MS-222 at 80 mg L^{-1} water for longer than 10 minutes, which includes time for "knockdown" or induction, to weigh and measure fish, to transfer to surgical cradle, and to conduct surgery. If this time limit is exceeded, the fish is to be released without surgical implantation, thereby increasing the number of fish handled and held at the juvenile bypass facilities for FCRPS survival studies. The CBSPSC expressed concern that the recommended 10-minute maximum exposure to MS-222 could be too long for a juvenile salmonid, resulting in increased behavior and physiological costs, and/or decreased survival. These undesirable results are further problematic because the assumptions of survival studies require that fish be representative of fish in the river that have not been subjected to handling, holding, and surgical implantation. Thus, there is a need to determine the amount of time fish can be held in knockdown anesthetic without negatively influencing their survival and thus biasing fish passage survival models.

1.2 Report Contents and Organization

Study methods, materials, and results are described in the ensuing sections of this report. The results of this report complement the compliance monitoring study (Carlson et al. in prep) conducted by researchers at PNNL and the University of Washington (UW) for the USACE. References cited in the text are listed in the final section.

2.0 Methods and Materials

The study, conducted in April 2010, involved the acquisition of fish, surgical implantation of transmitters, sampling techniques, and statistical analysis, as described below.

2.1 Fish Acquisition

YCH (wet weight [WW] 41.8 ± 10.4 g, fork length [FL] 150.5 ± 10.4 mm [mean ± SD, n = 292) raised from eggs and reared at PNNL Aquatics Research Laboratory facilities (Richland, Washington) were used for this study. Fish were housed in two 890-L circular fiberglass tanks supplied with aerated well water at 15°C and held on a 12-hour light to 12-hour dark photoperiod during the acclimation period. During the experimental period, fish were held in three 890-L circular fiberglass tanks with aerated well water chilled to 15°C and held on a 12-hour light to 12-hour dark photoperiod. Fish were fed daily (1.5% of the mean body weight) with BioDiet pellets (Bio-Oregon, Oregon). The tanks were cleaned twice a day to minimize food and fungal accumulation. All methods were approved by the Institutional Animal Care and Use Committee (IACUC Protocol # 2010-08).

2.2 Anesthetic Treatment and Sampling

Fish were randomly assigned to one of three groups—two control treatment groups and one time-anesthetic treatment group:

- anesthetic control (no anesthetic exposure, no surgical implantation; herein referred to as the AC group)
- surgical control (exposed to anesthesia and removed when reached Stage 4 anesthesia, no surgical implantation; herein referred to as the SC group)
- one of seven different time-anesthesia treatments where fish remained in the anesthetic for 0, 3, 5, 6, 9, 12, or 15 minutes beyond the time of Stage 4 induction, then were surgically implanted with an AT and PIT (herein referred to as KD group).

Fish in the 0-minute KD treatment group were immediately removed from a knockdown bath after reaching Stage 4 anesthesia. Fish in the 3- to 15-minute KD treatment groups remained in the anesthetic after reaching Stage 4 for their allotted time before being surgically implanted with a JSATS AT (Advance Telemetry Systems, Isanti, Minnesota) and PIT (Destron Technologies, St. Paul, Minnesota).

Control and KD treatment groups were then further assigned to one of four post-surgical implantation monitoring day groups (herein referred to as “post-implantation day treatment”):

- day of implantation (day 0)
- 1 day after implantation (day 1)
- 7 days after implantation (day 7)
- 14 days after implantation (day 14).

Day 0 fish were lethally sampled on the same day as the anesthetic treatment, while day 1, 7, and 14 fish were held until their respective days for sampling and termination. Fish were grouped in three experimental holding tanks by treatment "post-implantation day" assignment. The controls (both AC and SC), KD treatment groups, and post-implantation day treatment groups by the number of fish for each group are summarized in Table 2.1.

Table 2.1. The Anesthetic Treatment and Number of Fish Designated to Each Treatment Time and Day

Days	Anesthetic Control	Surgical Control	Lag Time from Stage 4 Anesthesia until Implantation (min)							# Fish Used
			0	3	5	6	9	12	15	
0	5	0	10	10	10	10	10	10	10	75
1	0	5	10	10	10	10	10	10	10	75
7	0	5	10	10	10	10	10	10	10	75
14	0	5	10	10	10	10	10	10	10	75

2.3 Surgical Implantation of Fish

Two anesthesiologists and two surgeons performed "knockdowns" and surgeries. Each surgery was standardized to take approximately 3 minutes. Surgical control and KD treatment groups (excluding the AC group) were anesthetized and handled. For these groups, a buffered anesthetic was prepared using aerated well water and MS-222, 80 mg L^{-1}. Prior to surgical implantation, fish were placed in buckets of anesthetic until loss of equilibrium (Stage 4; Summerfelt and Smith 1990). Anesthetized fish were immediately weighed, measured, assigned tags (except control fish), and both flanks were photographed to document gross observable fish condition. When properly anesthetized, fish receiving surgical implants were placed on the surgery table and given a maintenance anesthetic dose (well water containing MS-222, 40 mg L^{-1}) through silicone rubber tubing from a gravity-fed bucket. The surgeon controlled the exact dose during the procedure by mixing well water with the maintenance anesthetic water. With the fish facing ventral side up, a 5- to 7-mm incision (Micro-Unitome knife, 3-mm blade, Becton, Dickinson and Company, Franklin Lakes, New Jersey), was made along the linea alba between the pectoral fin and pelvic girdle. A JSATS micro-AT (12 mm long, 5.2 mm wide, 3.8 mm high, and weighing 0.43 g in air, 0.14 mL volume), and PIT (12.5 mm long, 2.1 mm wide, and weighing 0.10 g in air, 0.04 mL volume) were then inserted into the coelomic cavity through the incision. The incision was closed with two simple interrupted sutures using a 2×2×2×2 wrap knot pattern as described by Deters et al. (2010) for monofilament sutures with 5-0 Monocryl™ sutures. Post-surgery, fish were placed into 5-gal perforated recovery buckets (five fish per bucket) with fresh aerated well water and monitored to ensure they recovered equilibrium. The density of fish for each bucket did not exceed 15 kg/m^3. The fish were then released into larger holding tanks supplied with flow-through aerated well water. Fish were assigned to tanks by post-implantation sampling day and held until they were lethally sampled. Fish from post-implantation day 0 treatment were not placed into freshwater tanks for recovery, but were immediately sampled after the incision was closed and freshwater turned on fully; then they were euthanized. AC fish were placed in an ice bath for 2 minutes followed by a spinal transaction, posterior to the head, for euthanasia following established techniques by Wilson et al. (2009).

2.4 Euthanasia and Sampling

All treatment and SC fish were euthanized in a high dose of MS-222 (250 mg L^{-1}). After euthanasia (regardless of control or KD group), blood samples (0.4 mL) were collected from the caudal vein using a 21-G needle and 1-mL syringe containing 0.05 mL of sodium heparin. Blood samples were dispensed (with needle removed) into a polypropylene centrifuge tube and held on ice (estimated ≤ 5 minutes) for blood analyte analyses.

2.5 Physiological Measurements

Blood Na^+, K^+, Ca^{2+}, and pH levels were measured using an EasyLyte analyzer (Medica Corporation, Bedford, Massachusetts) with the internal calibration; single-point eternal standard verification for sodium, potassium, and calcium; and three-point external standard verification of pH. Quality assurance/quality control measures were consistently applied each day before sampling and throughout the day. The measurements were carried out according to validated standard operating procedures. Samples were centrifuged at 3,000 *g* for 10 minutes at 4°C, and the plasma was collected and stored at -80°C for subsequent cortisol analysis.

Plasma cortisol concentrations were determined by competitive plasma cortisol expression enzyme immunoassay (EIA) following the manufacturers protocol (Cayman Chemical Company, Ann Arbor, Michigan). Plasma samples were not purified prior to cortisol analysis because the preliminary tests indicated there was little to no interference from contaminants. Plasma was diluted 100 fold using EIA buffer, determined by preliminary tests. Plasma samples reading >80% bound cortisol were diluted 150 fold and retested. Samples were analyzed in triplicate (BioTek PowerWave HT, BioTek Inc.; set at 405 nm) and each assay plate included samples from all sampling times in a randomized order to minimize bias caused by interassay variation.

2.6 Statistical Analyses

Length and weight were regressed to ensure that they would not be confounding factors when pooling days in the rest of the analyses. Independent variables (AC, SC, KD, post-exposure implantation day groups; anesthetic indicator, surgical indicator, and day indicator) were examined by dependent measures of stress (blood analytes and pH, plasma cortisol, mortality). "Anesthetic Indicator" is an indicator of fish exposed to anesthetic and fish not exposed to anesthetic (Yes = fish exposed to anesthetic, No = fish not exposed to anesthetic). "Surgical Indicator" is an indicator of fish exposed to surgery and fish not exposed to surgery (Yes = fish exposed to surgery, No = fish not exposed to surgery). "Day Indicator" is an indicator of fish at day 0 and fish at days 1, 7, and 14 (Yes = fish at day 0, No = fish at day 1, 7, or 14). Normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test) were assessed for each test. If the normality and homoscedasticity assumptions were satisfied, the dependent variables were modeled using generalized linear models based on log link analysis. Analysis of deviance (ANODEV) was used in modeling the data and testing hypotheses. When a difference was detected ($P < 0.05$) and when appropriate, Tukey's honestly significant difference test was applied to identify which treatments were significantly different. If the conditions for analysis of variance were not satisfied, the non-parametric Kruskal-Wallis test was used (Zar 1996). Linear regression analysis was used to examine the relationship between KD treatment groups and survivorship. All tests were

considered significant at $P < 0.05$ and conducted using JMP® (Version 80, Cary, North Carolina). The small sample sizes and individual variation likely interfered with our ability to see significant differences at $\alpha = 0.05$.

3.0 Results

The ensuing sections describe data corrections needed during the study and the results related to fish size variability, mortalities, blood analyte profiles, and blood biochemistry profiles. The sections are based on the questions asked of the data: data corrections, fish size variability, mortalities, day 0 AC and KD treatments, day 0 within KD treatments (excluding controls), day and KD treatments, and day and KD treatments (excluding controls).

3.1 Data Corrections

During the monitoring period, eight fish were removed from the analysis (Table 3.1). These fish had tags incorrectly recorded or were found to have been outside of the tank for an undetermined period of time.

Table 3.1. Final Numbers of Fish per Treatment. Fish were not identifiable from lost PIT or AT tags.

Days	Anesthetic Control	Surgical Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)							Total # of Samples
			0	3	5	6	9	12	15	
0	5	0	10	10	10	10	10	9	9	73
1	0	5	10	10	10	9	10	10	9	73
7	0	5	10	10	10	10	10	10	10	75
14	0	5	9	10	9	9	9	10	10	71

The EasyLyte analyzer required samples to be aspirated from a needle inserted into the sampling vial. If the sample was clotted or pulled out of the aspirator too quickly, the analyzer would not measure the analytes. To ensure there was enough blood for the biochemistry panel, samples were analyzed only once using the EasyLyte analyzer. The final sample numbers used in the analyte blood data are provided in Table 3.2.

Table 3.2. Final Number of Samples per Treatment for Analyte Blood Profile Analyses (Na^+, K^+, Ca^{2+})

Days	Anesthetic Control	Surgical Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)							Total # of Samples
			0	3	5	6	9	12	15	
0	5	0	9	6	8	9	9	5	4	55
1	0	4	8	10	10	8	5	9	9	63
7	0	3	3	7	6	7	8	5	6	45
14	0	4	4	7	5	2	2	5	4	33

For the biochemistry blood profile, the EIA technique is based on competitive binding and quantified using a colorimetric plate reader. Blood samples that were too “red” (pigmented) from lysed red blood

cells could not be analyzed. If samples were clotted, the plasma sample could not be broken down for EIA analyses. The final sample numbers used in the biochemistry blood data are provided in Table 3.3.

Table 3.3. Final Number of Samples per Treatment for Biochemistry Blood Profile Analyses (pH, cortisol)

Days	Anesthetic Control	Surgical Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)							Total # of Samples
			0	3	5	6	9	12	15	
0	4	0	9	10	10	10	10	8	9	70
1	0	4	10	10	10	9	6	10	9	68
7	0	5	10	10	10	10	9	10	10	75
14	0	5	9	10	9	9	10	10	9	71

3.2 Size Variability

At the time of tagging, YCH FLs and WWs ranged from 127 to 193 mm (median 150 mm) and 20.4 to 100.8 g (median 40.1 g; Table 3.4). For each KD by day treatment, WW significantly predicted FL (all $P < 0.03$; Table 3.4). Similarly, for each day treatment (across KD treatments), WW significantly predicted FL (all $P < 0.001$; Table 3.4). There were no significant differences in fish weight among day treatments (all $P \geq 0.286$; Table 3.5), allowing the fish to be pooled for later analyses.

Table 3.4. Fork Length (FL, mm) and Wet Weight (WW, g) Means, Standard Deviations, and Regression Relationships for Fish Assigned to a KD and Day Treatment Groups

Day	Time	FL (mm)	WW (g)	N	Intercept	Slope	r^2	*F*	*P*
0	All Times	148.8 ± 10.2	42.3 ± 10.1	65	109.48	0.931	0.8	334	< 0.001
	0	148.4 ± 9.5	41.6 ± 10.3	10	111.68	0.882	0.9	80.5	< 0.001
	3	145.7 ± 12.0	38.7 ± 10.2	10	103.71	1.086	0.9	44.8	< 0.001
	5	152.6 ± 10.0	46.6 ± 10.9	10	116.57	0.774	0.7	17.9	0.003
	6	147.4 ± 11.0	40.3 ± 9.2	10	102.36	1.12	0.9	60	< 0.001
	9	147.9 ± 7.3	40.7 ± 6.8	10	111.46	0.894	0.7	19.2	< 0.002
	12	148.0 ± 11.9	39.7 ± 11.0	8	106.7	0.986	1	133	< 0.001
	15	152.9 ± 10.9	47.7 ±11.6	7	111.04	0.877	0.9	31.5	0.003
1	All Times	151.2 ± 9.9	41.4 ± 9.9	68	111.58	0.957	0.9	693	< 0.001
	0	150.0 ± 8.1	40.3 ± 9.2	10	115.87	0.846	0.9	83.5	< 0.001
	3	152.0 ± 10.8	42.5 ± 11.3	10	122.26	0.935	1	193	< 0.001
	5	150.6 ± 11.7	41.9 ± 10.2	10	103.66	1.121	1	169	< 0.001
	6	154.6 ± 8.2	44.2 ± 9.2	9	117.16	0.846	0.9	65	< 0.001
	9	144.9 ± 10.3	35.3 ± 7.5	10	98.1	1.325	0.9	83.5	< 0.001
	12	149.9 ± 9.3	44.2 ± 13.0	10	117.86	0.807	0.9	75.2	< 0.001
	15	157.1 ± 8.3	46.4 ± 9.3	9	117.44	0.855	0.9	80	< 0.001

Table 3.4. (contd)

Day	Time	FL (mm)	WW (g)	N	Intercept	Slope	r^2	*F*	*P*
7	All Times	150.4 ± 9.5	40.3 ± 8.8	70	109.54	1.014	0.9	495	< 0.001
	0	149.4 ± 6.8	37.9 ± 5.6	10	107.89	1.1	0.8	34.7	0.004
	3	153.4 ± 11.8	42.0 ± 9.5	10	103.27	1.194	0.9	114	< 0.001
	5	151.0 ± 9.9	41.0 ± 8.7	10	105.44	1.11	1	175	< 0.001
	6	149.7 ± 3.8	38.7 ± 2.4	10	97.81	1.34	0.7	19.2	< 0.001
	9	152.6 ± 8.1	41.0 ± 6.6	10	105.32	1.152	0.9	80.9	< 0.001
	12	150.7 ± 13.0	44.2 ± 13.0	10	108.41	0.957	0.9	95.1	< 0.001
	15	145.9 ± 11.0	37.1 ± 11.4	10	111.36	0.932	0.9	120	< 0.001
14	All Times	151.6 ± 11.8	43.2 ± 12.6	66	112.9	0.894	0.9	639	< 0.001
	0	150.2 ± 13.6	43.4 ± 15.1	9	111.87	0.883	1	172	< 0.001
	3	151.9 ± 10.8	42.1 ± 11.3	10	112.33	0.94	1	225	< 0.001
	5	154.1 ± 6.1	44.4 ± 7.0	9	116.87	0.838	0.9	73.2	< 0.001
	6	155.9 ± 4.2	46.8 ± 7.4	9	136.61	0.412	0.5	7.42	0.03
	9	145.8 ± 10.6	41.9 ± 11.8	9	100.59	1.173	1	145	< 0.001
	12	150.3 ± 18.5	43.9 ± 22.2	10	114.58	0.814	1	168	< 0.001
	15	152.9 ± 13.2	43.5 ± 11.3	10	103.18	1.144	1	184	< 0.001

Table 3.5. Analysis of Variation Results for Fish Wet Weight (g) Assigned to Time and Post-Exposure Day Treatment Groups

Day	Mean	Std Dev	N	df	*F*	*P*
0	42.3	10.1	65	6, 58	0.955	0.464
1	41.4	9.9	68	6, 62	1.267	0.286
7	40.3	8.8	70	6, 63	0.813	0.564
14	43.2	12.6	66	6, 59	0.34	0.913

3.3 Mortalities

Contrary to our initial hypothesis, there were no mortalities during the experiment for any KD or post-implantation day treatment groups.

3.4 Day 0: Anesthetic Controls vs. KD Treatments

Post-implantation day 0 fish (i.e., tag implanted, incisions closed, blood samples taken immediately) and the AC YCH (i.e., YCH not exposed to MS-222 nor implanted with tags) were first tested for outliers. On day 0, the mean sodium concentrations for AC YCH was 156.9 ± 3.0 mmol L^{-1}, and the sodium concentrations for YCH in the KD treatments ranged from 114.1 to 156.7 mmol L^{-1} (Figure 3.2, Table 3.7). For AC YCH, calcium concentrations were 1.49 ± 0.31 mmol L^{-1}, and the calcium concentrations for YCH in the KD treatments ranged from 0.08 to 0.78 mmol L^{-1} on day 0 (Figure 3.3, Table 3.8). The mean potassium concentrations for AC YCH were 3.8 ± 0.4 mmol L^{-1}, and the potassium concentrations for YCH in the KD treatments ranged from 2.2 to 5.0 mmol L^{-1} (Figure 3.4, Table 3.9).

pH values for AC YCH were 7.16 ± 0.04, and the pH values for YCH in the KD treatments ranged from 7.17 to 7.28 on day 0 (Figure 3.5, Table 3.10). Lastly, for AC YCH, cortisol concentrations were 0.07 ± 0.02 µg L^{-1} and the cortisol concentrations for YCH in the KD treatments ranged from 0.09 to 0.14 µg L^{-1} on day 0 (Figure 3.6, Table 3.11).

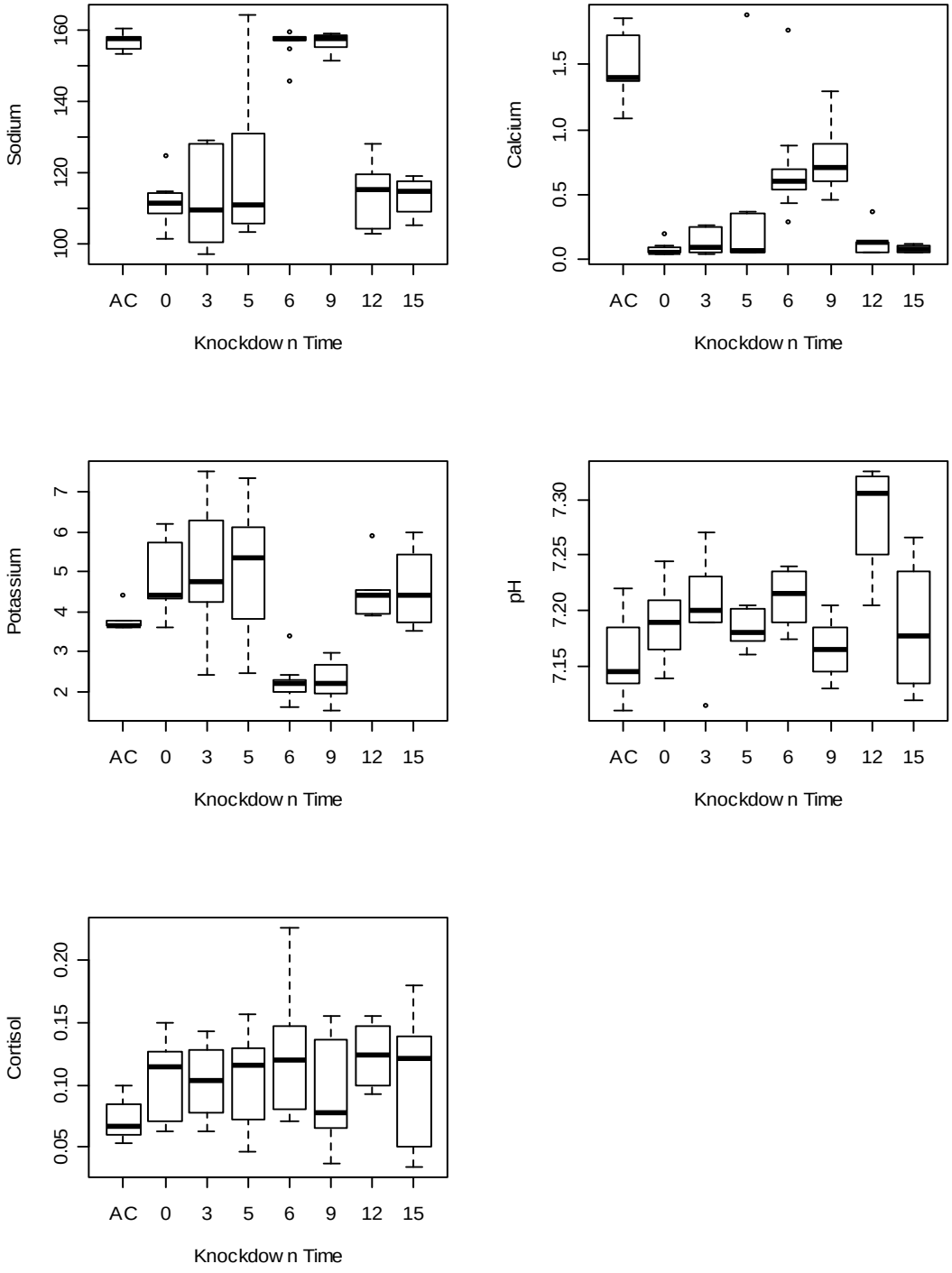

Figure 3.1. Box Plots and Outlier Analyses for Blood Analytes and Biochemistry Measures, Including AC on Day 0

Table 3.6. Analysis of Deviance Results for All Five Response Variables for Day 0. Anesthetic indicator tests for a difference between YCH that received anesthetic and those that were not exposed to the anesthetic (AC; no surgical control).

Response	Df	Deviance	Resid.Df	Resid.Dev	*F*	*Pr*(>F)
Sodium						
Null	54	29206				
Anesthetic Indicator	1	3389.2	53	25817	6.9578	0.0109[(a)]
Calcium						
Null	54	15.1182				
Anesthetic Indicator	1	5.5863	53	9.5319	31.061	<0.0001[(a)]
Potassium						
Null	54	130.71				
Anesthetic Indicator	1	0.042592	53	130.66	0.0173	0.8959
pH						
Null	54	0.11969				
Anesthetic Indicator	1	0.0069847	53	0.11271	3.2845	0.0756
Cortisol						
Null	69	0.10808				
Anesthetic Indicator	1	0.005151	68	0.10293	3.4029	0.0694

(a) Tests that are significant between the AC and the KD treatment exposed fish.

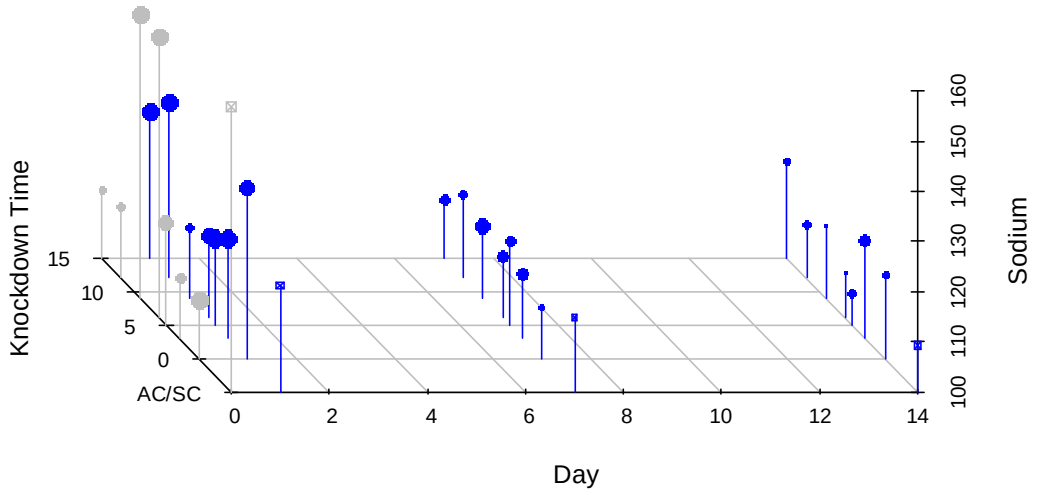

Figure 3.2. 3-D Plot of Blood Na^+ (mmol L^{-1}) by KD and Post-Implantation Day. Grey circles indicate day 0. The grey square on day 0 indicates AC and blue squares on days 1, 7, and 14 indicate SC. Blue circles indicate day 1, 7, and 14 KD treatments. The size of the circles corresponds to the sample size for each KD and day combination.

On day 0, AC YCH had significantly higher sodium and calcium concentrations (both $P \leq 0.01$) when compared to exposed YCH in the other KD treatments (Figure 3.1, Table 3.6). When examining where the differences arose, AC YCH sodium concentrations were not significantly different from the 6- and 9-minute anesthetic KD treatments (Figure 3.2, Table 3.7). AC YCH calcium concentrations were

significantly greater than the 0-, 3-, 5-, 6-, 9-, 12-, and 15-minute KD treatments (Figure 3.1, Table 3.6). The 6- and 9-minute KD treatments had significantly greater calcium concentrations than the 0-, 3-, 5-, 12-, and 15-minute KD treatments, although as mentioned, they were significantly lower than the AC concentrations (Table 3.8). The concentrations of potassium in the AC fish were non-significantly less than those in the 0-, 3-, 5-, 12-, and 15-minute KD treatments, but they were significantly greater than the 6- and 9-minute KD treatments (Figure 3.1, Table 3.6, Table 3.9). The AC YCH on day 0 had lower, but not significantly lower, pH and cortisol levels when compared to the YCH exposed to anesthesia (Figure 3.1, Figure 3.6, Table 3.6, Table 3.10, Table 3.11).

Table 3.7. Comparison of the Blood Na^+ (mmol L^{-1}) Analyte Profile for Each KD and Day Treatment

Day	Anesthetic Control	Surgery Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)						
			0	3	5	6	9	12	15
0	156.9 ± 3.0*	--	111.5 ± 6.5	112.3 ± 13.9	120.3 ± 21.0	156.2 ± 4.1[(a)]	156.7 ± 2.5[(a)]	114.1 ± 10.6	114.1 ± 5.2
1	--	121.2 ± 19.9	134.0 ± 22.3[(a)]	121.4 ± 32.5	117.1 ± 26.5	116.5 ± 25.8	114.0 ± 20.8	135.1 ± 14.7[(a)]	129.8 ± 22.0[(a)]
7	--	114.9 ± 7.3	110.0 ± 4.9	112.7 ± 6.8	116.7 ± 18.7	112.2 ± 4.7	114.5 ± 6.7	116.8 ± 5.6	111.7 ± 9.3
14	--	109.2 ± 9.9	116.9 ± 5.0	119.3 ± 13.0	106.7 ± 1.8	109.2 ± 1.7	111.4 ± 7.7	110.7 ± 7.9	119.3 ± 11.6

(a) Significant differences between treatments within a day.

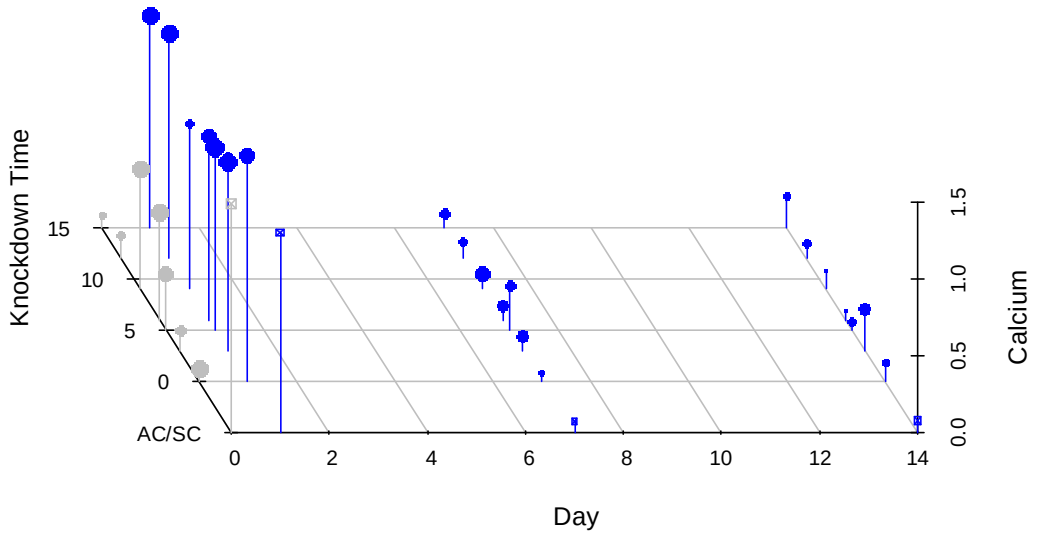

Figure 3.3. 3-D Plot of Blood Ca+ (mmol L-1) by KD and Post-Implantation Day. Grey circles indicate day 0. The grey square on day 0 indicates AC and blue squares on days 1, 7, and 14 indicate SC. Blue circles indicate day 1, 7, and 14 KD treatments. The size of the circles corresponds to the sample size for each KD and day combination.

Table 3.8. Comparison of the Blood Ca2+ (mmol L-1) Analyte Profile for Each KD and Day Treatment

Day	Anesthetic Control	Surgery Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)						
			0	3	5	6	9	12	15
0	1.49 ± 0.31[(a)]	--	0.08 ± 0.05	0.13 ± 0.1	0.36 ± 0.63	0.70 ± 0.43[(b)]	0.78 ± 0.27[(b)]	0.15 ± 0.13	0.09 ± 0.03
1	--	1.30 ± 0.44	1.46 ± 0.4	1.25 ± 0.56	1.19 ± 052	1.19 ± 0.49	1.08 ± 0.42[(a)]	1.47 ± 0.29	1.40 ± 0.40
7	--	0.07 ± 0.04	0.05 ± 0.01	0.09 ± 0.06	0.09 ± 0.05	0.09 ± 0.05	0.10 ± 0.05	0.11 ± 0.06	0.10 ± 0.07
14	--	0.08 ± 0.06	0.13 ± 0.05	0.27 ± 0.26	0.06 ± 0.01	0.06 ± 0.0	0.12 ± 0.05	0.09 ± 0.04	0.21 ± 0.23

(Superscript letters) Significant differences between treatments within a day.

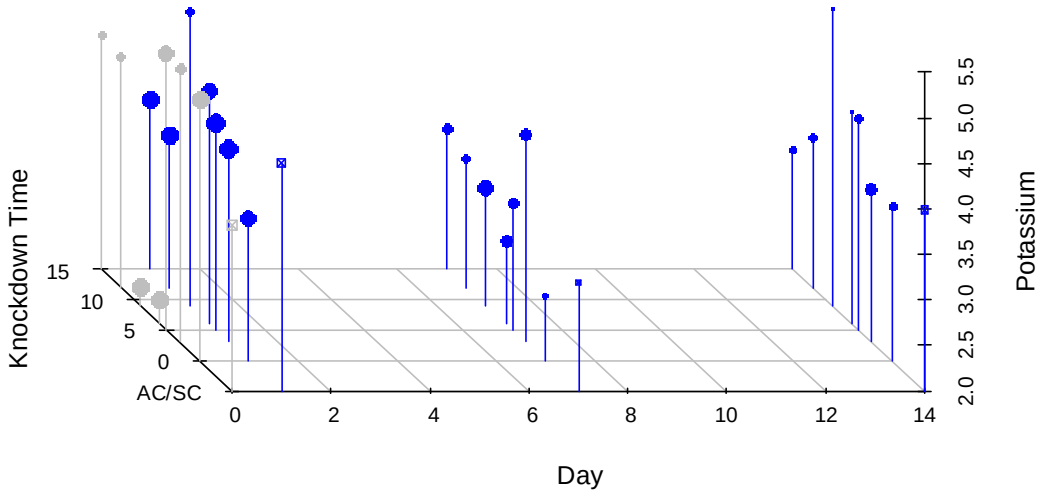

Figure 3.4. 3-D Plot of Blood K+ (mmol L-1) by KD and Post-Implantation Day. Grey circles indicate day 0. The grey square on day 0 indicates AC and blue squares on days 1, 7, and 14 indicate SC. Blue circles indicate day 1, 7, and 14 KD treatments. The size of the circles corresponds to the sample size for each KD and day combination.

Table 3.9. Comparison of the Blood K+ (mmol L-1) Analyte Profile for Each KD and Day Treatment

Day	Anesthetic Control	Surgery Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)						
			0	3	5	6	9	12	15
0	3.8 ± 0.4	--	4.9 ± 0.9	5.0 ± 1.8	5.0 ± 1.6	2.3 ± 0.5[(a)]	2.2 ± 0.5[(a)]	4.5 ± 0.8	4.6 ± 0.9
1	--	4.5 ± 1.8[(a)]	3.6 ± 1.7	4.0 ± 1.8	4.3 ± 1.5	4.6 ± 1.3[(a)]	5.2 ± 3.1[(a)]	3.7 ± 0.8	3.7 ± 1.7
7	--	3.29 ± 0.68	2.71 ± 0.28	4.28 ± 0.49	3.40 ± 0.50	2.90 ± 0.40	3.3 ± 0.8	3.41 ± 0.84	3.56 ± 0.67
14	--	3.99 ± 1.23	3.71 ± 0.88	3.67 ± 0.44	4.33 ± 1.17	4.33 ± 1.07	5.26 ± 0.44	3.65 ± 0.56	3.32 ± 0.89

(a) Significant differences between treatments within a day.

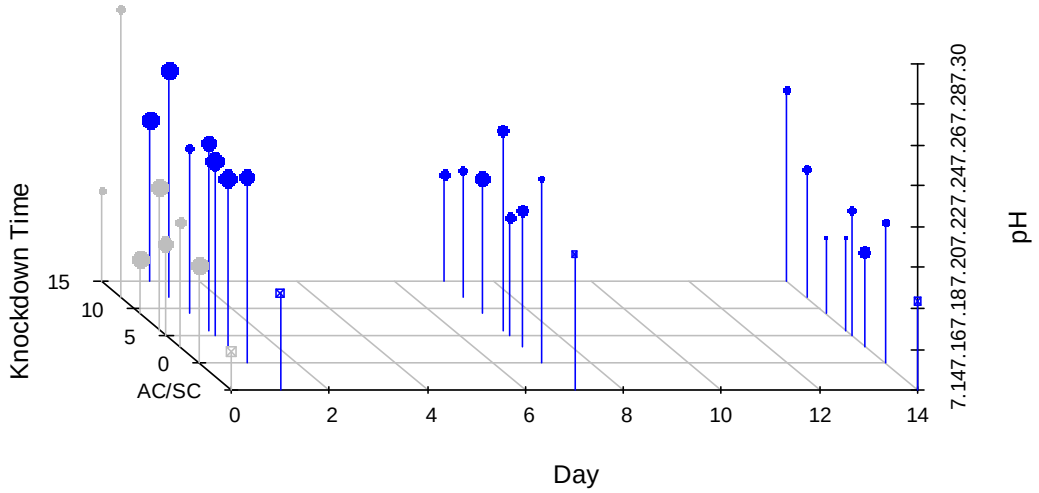

Figure 3.5. 3-D Plot of Blood pH by KD and Post-Implantation Day. Grey circles indicate day 0. The grey square on day 0 indicates AC and blue squares on days 1, 7, and 14 indicate SC. Blue circles indicate day 1, 7, and 14 KD treatments. The size of the circles corresponds to the sample size for each KD and day combination.

Table 3.10. Comparison of the Blood pH Biochemistry Profile for Each KD and Day Treatment

Day	Anesthetic Control	Surgery Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)						
			0	3	5	6	9	12	15
0	7.16 ± 0.04	--	7.19 ± 0.03	7.20 ± 0.05	7.18 ± 0.02	7.21 ± 0.03	7.17 ± 0.03	7.28 ± 0.05	7.20 ± 0.06
1	--	7.19 ± 0.13	7.23 ± 0.03	7.22 ± 0.04	7.23 ± 0.04	7.23 ± 0.05	7.22 ± 0.06	7.25 ± 0.05	7.22 ± 0.04
7	--	7.21 ± 0.01	7.23 ± 0.03	7.21 ± 0.02	7.20 ± 0.03	7.24 ± 0.04	7.21 ± 0.04	7.21 ± 0.01	7.19 ± 0.03
14	--	7.18 ± 0.04	7.21 ± 0.02	7.19 ± 0.03	7.21 ± 0.03	7.19 ± 0.01	7.18 ± 0.02	7.20 ± 0.05	7.23 ± 0.06

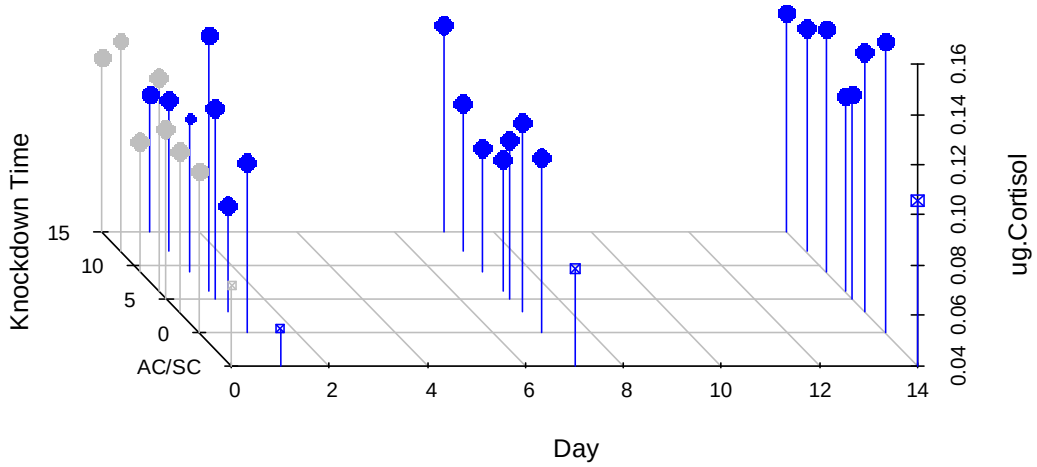

Figure 3.6. 3-D Plot of Cortisol (µg L^{-1}) by KD and Post-Implantation Day. Grey circles indicate day 0. The grey square on day 0 indicates AC and blue squares on days 1, 7, and 14 indicate SC. Blue circles indicate day 1, 7, and 14 KD treatments. The size of the circles corresponds to the sample size for each KD and day combination.

Table 3.11. Comparison of the Plasma Cortisol Biochemistry Profiles for Each Day

Day	Anesthetic Control	Surgery Control	Lag Time from Stage 4 Anesthesia Until Implantation (min)					
			3	5	6	9	12	15
0	0.07 ± 0.02	--	0.10 ± 0.03	0.11 ± 0.04	0.12 ± 0.04	0.09 ± 0.04	0.14 ± 0.03	0.11 ± 0.05
1	--	0.06 ± 0.04	0.08 ± 0.04	0.15 ± 0.04	0.14 ± 0.06	0.10 ± 0.04	0.10 ± 0.05	0.09 ± 0.05
7	--	0.08 ± 0.03	0.12 ± 0.03	0.10 ± 0.05	0.09 ± 0.03	0.09 ± 0.04	0.10 ± 0.04	0.12 ± 0.05
14	--	0.09 ± 0.02	0.14 ± 0.03	0.12 ± 0.03	0.12 ± 0.05	0.14 ± 0.04	0.13 ± 0.05	0.13 ± 0.03

3.5 Day 0: KD Treatments Excluding Anesthetic Control

There were significant differences for sodium, calcium, potassium, or pH between KD (all $P \leq 0.001$; Figure 3.7, Table 3.12). These differences were generally visible in the boxplots (Figure 3.7), where the 6- and 9-minute KDs were consistently different from the other KDs resulting in non-monotonic response patterns. The pH of the 12-minute treatment group was significantly greater than the other KD treatments ($P < 0.003$). Cortisol levels were not significantly different between the KD treatment groups (all $P > 0.56$, Tables 3.12 and 3.13). No monotonic pattern was found between KD and the cortisol concentrations.

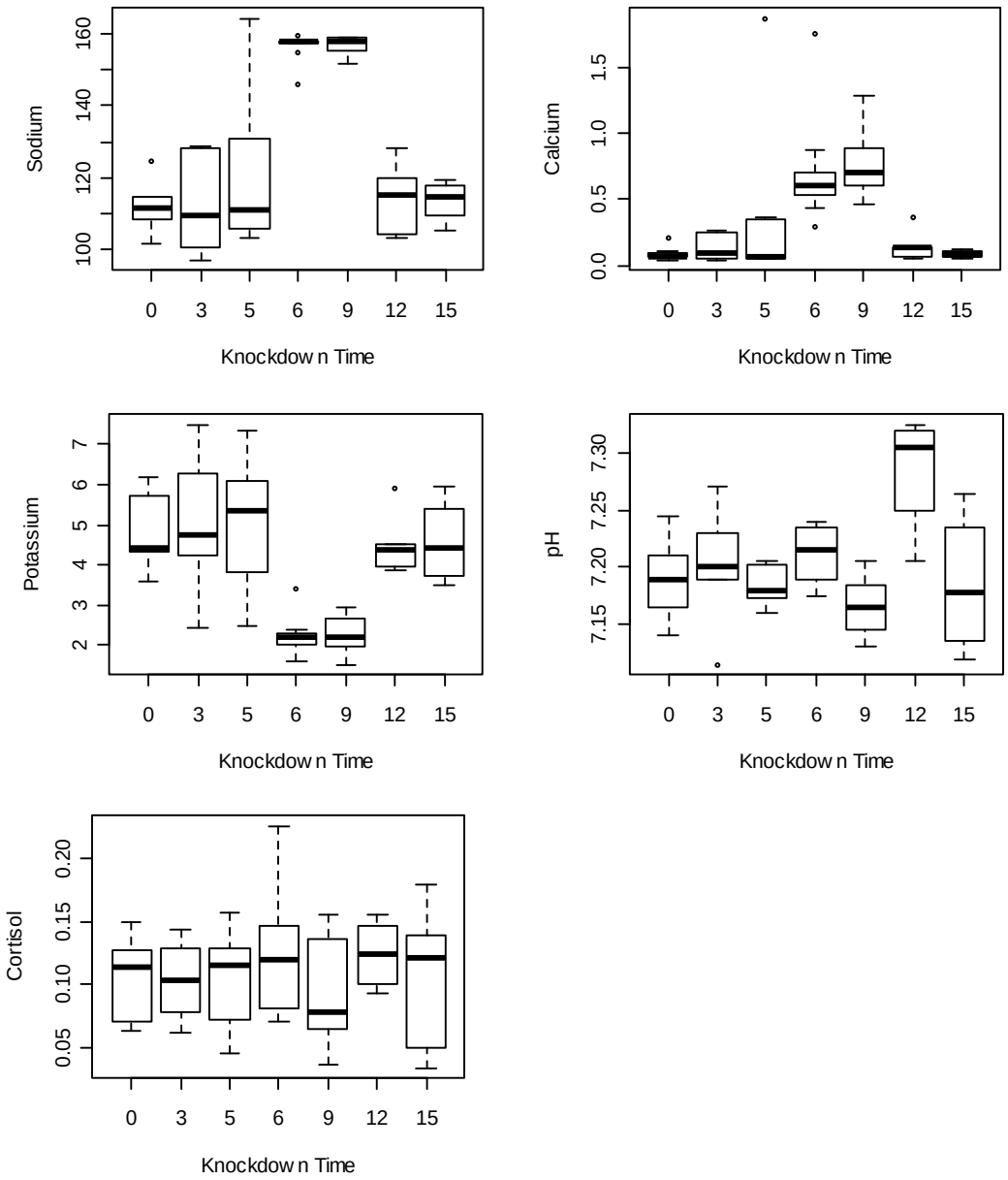

Figure 3.7. Box Plots for All Five Response Variables for Day 0. AC fish were removed from the analyses because analyses are focused on comparison of among KDs.

Table 3.12. ANODEV Results for the Five Response Variables on Day 0 for KD Treatment Groups

Response	Df	Deviance	Resid.Df	Resid.Dev	*F*	*Pr*(>F)
Sodium						
NULL	49	25781.7				
KD	6	20666	43	5115.9	28.95	<0.0001[(a)]
Calcium						
NULL	49	9.1542				
KD	6	4.1786	43	4.9756	6.0186	0.0001[(a)]
Potassium						
NULL	49	130.172				
KD	6	79.658	43	50.514	11.301	<0.0001[(a)]
pH						
NULL	49	0.10514				
KD	6	0.047848	43	0.05729	5.9854	0.0001[(a)]
Cortisol						
NULL	65	0.101748				
KD	6	0.0078524	59	0.093895	0.8224	0.5572

(a) Significant differences within day 0, between KDs.

Table 3.13. Comparison of the Blood Analyte and Biochemistry Profiles for All Treatment Days

	Treatment Days			
Measure	0	1	7	14
Blood Na^+ (mmol L^{-1})	131.8 ± 23.1[(a)]	122.7 ± 26.9[(a)]	113.8 ± 8.76	113.6 ± 9.64
Blood K^+ (mmol L^{-1})	3.92 ± 1.54[(a)]	4.08 ± 1.60[(a)]	3.90 ± 0.90	3.40 ± 0.90
Blood Ca^{2+} (mmol L^{-1})	0.37 ± 0.43[(b)]	1.30 ± 0.45[(a)]	0.12 ± 0.21	0.15 ± 0.17
Blood pH	7.21 ± 0.05	7.23 ± 0.04[(a)]	7.21 ± 0.03[(a)]	7.20 ± 0.04
Plasma Cortisol (μg L^{-1})	0.11 ± 0.04	0.11 ± 0.05	0.10 ± 0.04	0.13 ± 0.04[(a)]

(Superscript letters) indicate significant differences among days.

3.6 Post-Implantation Days 1, 7, and 14: Time Treatments Using Surgical Indicator Measures

Sodium, calcium, pH, and cortisol concentrations were significantly different ($P < 0.05$), while potassium was the only stress indicator measure not significantly different ($P > 0.05$), over days 1, 7, and 14 (Figures 3.8 to 3.12, Table 3.14, Table 3.15). For sodium, calcium, and pH, the level of stress indicator decreased from day 1 to day 14. For cortisol, the stress indicator went up from day 1 to day 14 (Figure 3.12). For cortisol, the Surgery Indicator was significant ($P = 0.0056$).

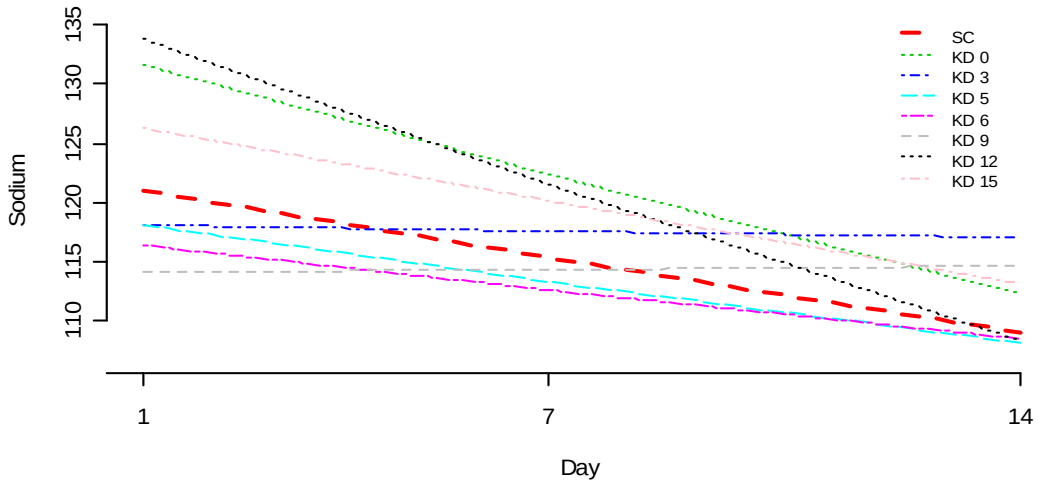

Figure 3.8. Plots Show the Predicted Values of Sodium over Days 1, 7, and 14. Each line represents a KD or surgical control. By comparing the line for SC (shown in red) with the other seven lines, we can visually compare the surgical control fish and fish that were surgically implanted with a tag.

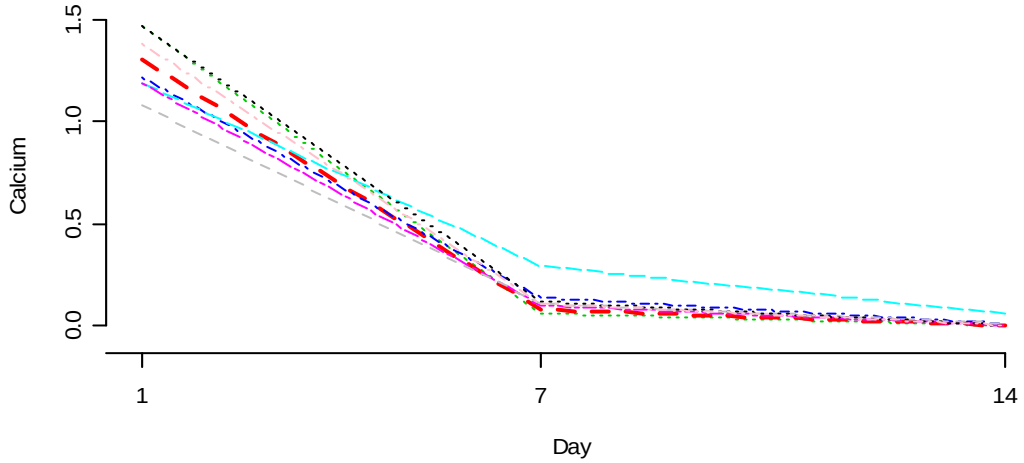

Figure 3.9. Plots Show the Predicted Values of Calcium over Days 1, 7, and 14. Each line represents a KD or surgical control. By comparing the line for SC (shown in red) with the other seven lines, we can visually compare the surgical control fish and fish that were surgically implanted with a tag.

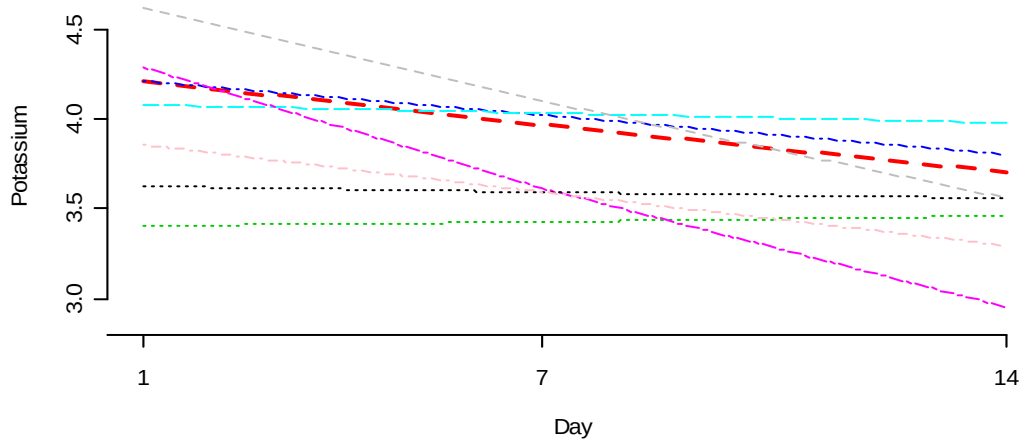

Figure 3.10. Plots Show the Predicted Values of Potassium over Days 1, 7, and 14. Each line represents a KD or surgical control. By comparing the line for SC (shown in red) with the other seven lines, we can visually compare the surgical control fish and fish that were surgically implanted with a tag.

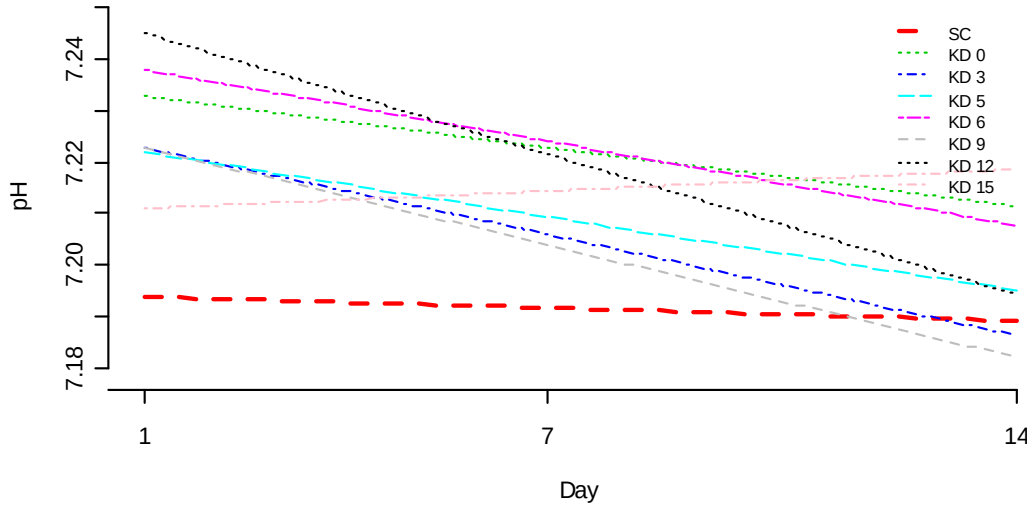

Figure 3.11. Plots Show the Predicted Values of pH over Days 1, 7, and 14. Each line represents a KD or surgical control. By comparing the line for SC (shown in red) with the other seven lines, we can visually compare the surgical control fish and fish that were surgically implanted with a tag.

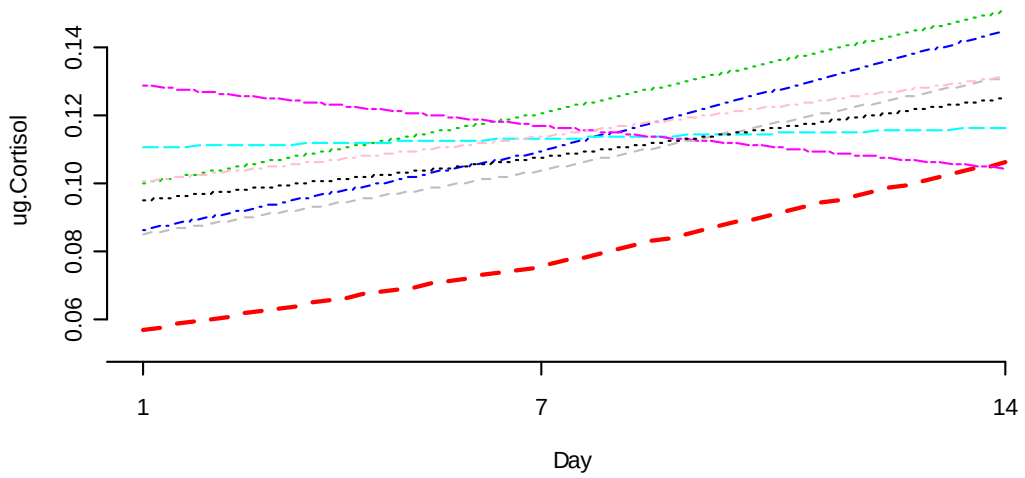

Figure 3.12. Plots Show the Predicted Values of Cortisol over Days 1, 7, and 14. Each line represents a KD or surgical control. By comparing the line for SC (shown in red) with the other seven lines, we can visually compare the surgical control fish and fish that were surgically implanted with a tag.

Table 3.14. ANODEV Results for All Five Response Variables. The day parameter in the Response column tests whether there were differences in responses over days 1, 7, and 14. The Surgery Indicator parameter tests whether there are differences between surgery fish and surgical control fish.

Response	Df	Deviance	Resid.Df	Resid.Dev	*F*	Pr(>F)
Sodium						
NULL	140	47138				
Day	1	2823.12	139	44315	8.7367	0.0037[(a)]
Surgery Indicator	1	44.27	138	44271	0.138	0.7109
Calcium						
NULL	140	62.91				
Day	1	46.887	139	16.022	403.8127	<0.0001[(a)]
Surgery Indicator	1	0	138	16.022	0.0004	0.9841
Potassium						
NULL	140	217.07				
Day	1	2.72518	139	214.34	1.7441	0.1888
Surgery Indicator	1	0.25734	138	214.09	0.1659	0.6844
pH						
NULL	140	0.28093				
Day	1	0.01808	139	0.26286	9.6691	0.0023[(a)]
Surgery Indicator	1	0.004815	138	0.25804	2.5751	0.1108
Cortisol						
NULL	213	0.43137				
Day	1	0.029559	212	0.40181	16.0988	<0.0001[(a)]
Surgery Indicator	1	0.014398	211	0.38741	7.8416	0.0056

(a) Significant differences between days 1, 7, 14.

Table 3.15. Analysis of Deviance Results for All Five Response Variables. KD tests whether there were differences in response between each KD, over all days. The "Day Indicator" tests whether there were differences in response between day 0 and days 1, 7, and 14. The "Interaction Effect" tests whether the difference in stress indicator values at day 0 and days 1, 7, and 14 change over KD.

Response	Df	Deviance	Resid.Df	Resid.Dev	*F*	*P*r(>F)
Sodium						
NULL	179	75365				
KD	6	4835.6	173	70529	2.7895	0.0130[(a)]
Day Indicator	1	4244.4	172	66285	14.6909	0.0002[(a)]
Interaction Effect	6	18325.9	166	47959	10.5719	<0.0001[(a)]
Calcium						
NULL	179	70.337				
KD	6	0.3121	173	70.025	0.1399	0.9907
Day Indicator	1	2.9916	172	67.034	8.0464	0.0051[(a)]
Interaction Effect	6	5.3172	166	61.716	2.3836	0.0310[(a)]
Potassium						
NULL	179	328.66				
KD	6	22.385	173	306.27	2.5716	0.0208[(a)]
Day Indicator	1	2.232	172	304.04	1.5388	0.2165
Interaction Effect	6	63.215	166	240.83	7.2624	<0.0001[(a)]
pH						
NULL	179	0.33564				
KD	6	0.030438	173	0.3052	3.1572	0.0059[(a)]
Day Indicator	1	0.01054	172	0.29466	6.5593	0.0113[(a)]
Interaction Effect	6	0.027935	166	0.26673	2.8976	0.0103[(a)]
Cortisol						
NULL	264	0.50333				
KD	6	0.005417	258	0.49792	0.4623	0.8359
Day Indicator	1	0.0012826	257	0.49663	0.6568	0.4185
Interaction Effect	6	0.0064453	251	0.49019	0.55	0.7697

(a) Significant interactions.

3.7 Post-Exposure Days 1, 7, and 14 vs. Day 0 Across KD Treatments

The interaction of day and KD was significant for the response variables sodium, calcium, potassium, and pH. The effect of KD changed between day 0 and days 1, 7, and 14. For sodium, potassium, pH, and cortisol, the value of each Stress Indicator (predicted values) on days 0, 1, 7, and 14 changed in a non-monotonic pattern over KD (Figures 3.13, 3.15, 3.16, and 3.17). For calcium, the Stress Indicator (predicted value) for days 0, 1, 7, and 14, all moved in a monotonic pattern over KD, but that pattern was not consistent between days (Figure 3.14). The Stress Indicator values for sodium and calcium indicate lowest levels on day 14 in the 6 and 9 min treatment groups. Conversely, on day 14, the 9 min KD treatment group Stress Indicator values was elevated. Statistics were not conducted on the Stress Indicator values.

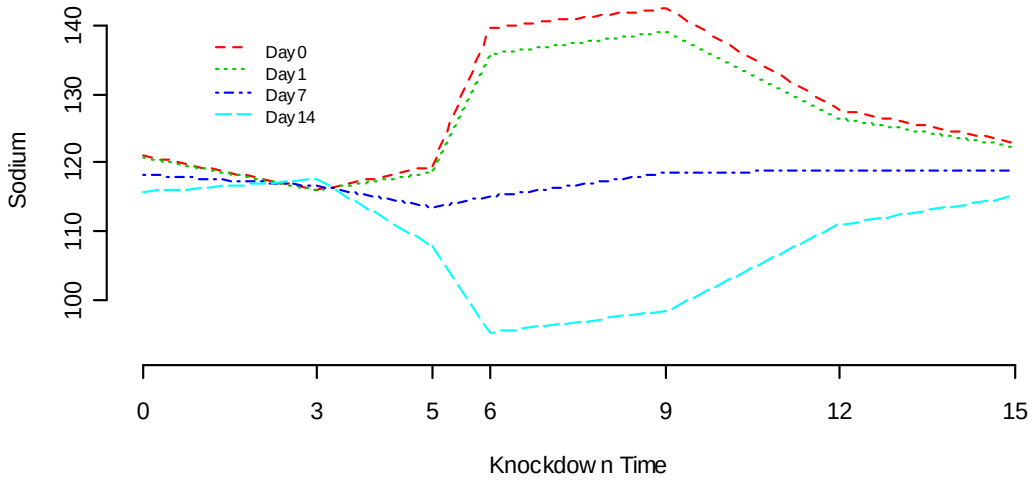

Figure 3.13. Plots Show the Predicted Values of Sodium over KDs Between 0 and 15 Minutes. Each line represents a different day post-surgery.

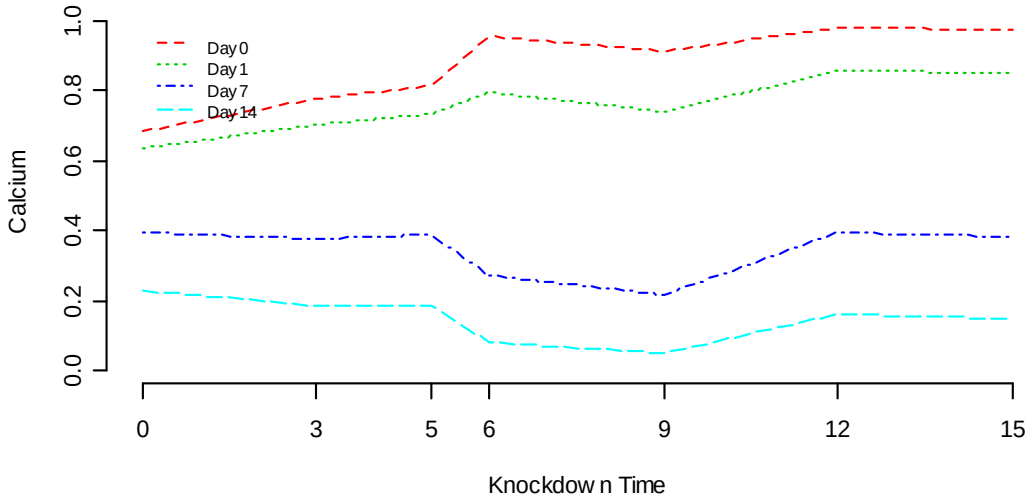

Figure 3.14. Plots Show the Predicted Values of Calcium over KDs Between 0 and 15 Minutes. Each line represents a different day post-surgery.

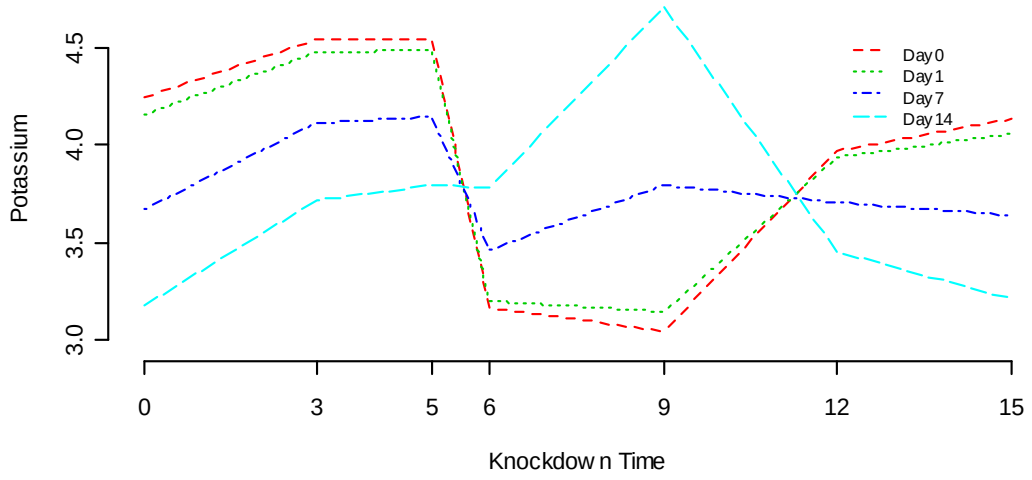

Figure 3.15. Plots Show the Predicted Values of Potassium over KDs Between 0 and 15 Minutes. Each line represents a different day post-surgery.

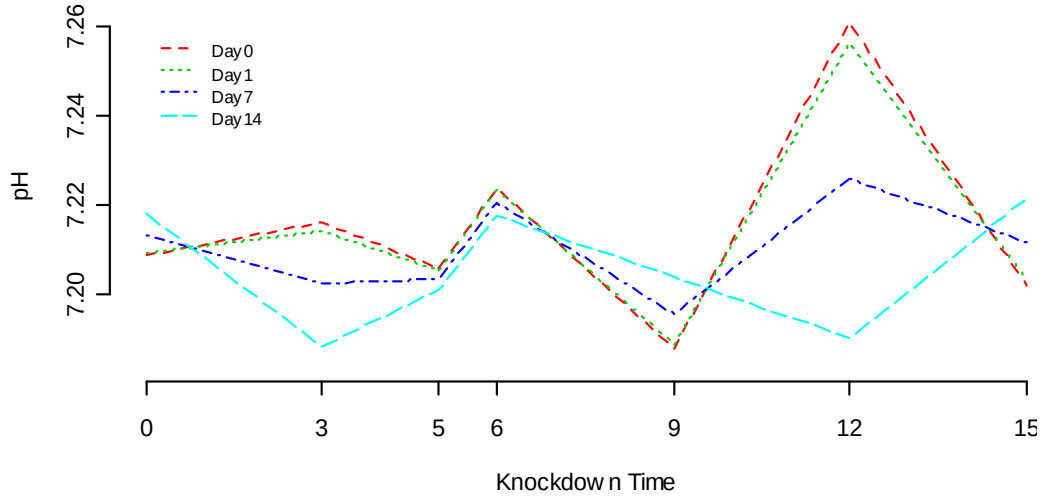

Figure 3.16. Plots Show the Predicted Values of pH over KDs Between 0 and 15 Minutes. Each line represents a different day post-surgery.

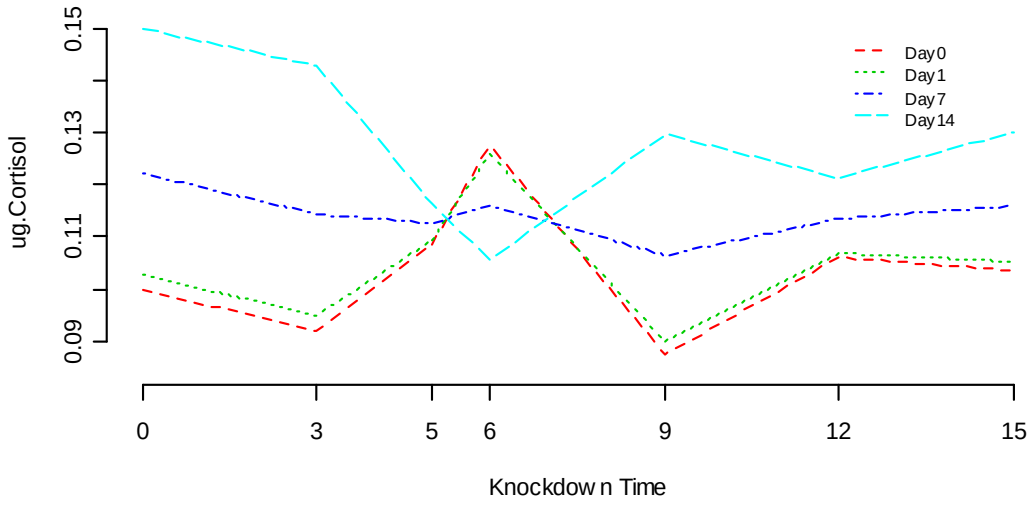

Figure 3.17. Plots Show the Predicted Values of Cortisol over KDs Between 0 and 15 Minutes. Each line represents a different day post-surgery.

4.0 Discussion

Routinely, anesthetics are used to reduce the stress of handling, invasive surgeries, and biological sampling through the modulation of the CNS system (e.g., sodium channels; Butterworth and Strichartz 1990; Arnolds et al. 2002). In addition, MS-222 is often used as an anesthetic before blood sampling to obtain resting or baseline levels of physiological measures. Conversely, prolonged exposure to anesthetics can induce stress, alter blood biochemistry, and even affect survival rates. Common measures for determining the effect of anesthetic stress are cortisol (Wagner et al. 2002; Congleton 2006; Zahl et al. 2011), partial pressure of blood gases, erythrocytes (Cho and Heath 2000), and plasma proteins, glucose, lactate, and ion (analyte) concentrations (Bourne 1984; Congleton 2006). The results from this study indicate that the long exposure periods and the surgery itself increase the overall stress response of juvenile salmonids. The fish exposed to MS-222 that did not undergo surgery had significantly lower stress levels as determined by the blood analytes. However, it was difficult to determine whether one KD exposure in Stage 4 was better than another KD exposure because the blood analytes and biochemistries varied within a day and did not produce any monotypic patterns. The 6- and 9-minute KD exposure groups analyte stress indictors by 14 days may indicate optimal exposure periods. YCH mortality did not occur as expected in the longer exposure times. Field mortalities may be related to sedation or the anesthetic process, YCH preexisting conditions, or fish that drifted past Stage 4 anesthesia. Over the duration of the study, we observed that juvenile YCH recovered from the anesthetic and surgical stress as the blood analytes and pH significantly decreased over the post-implantation days.

During the study, there were no mortalities during knockdown, surgical implantation, or on any recovery days. Based on the concerns expressed by the CBSPSC, mortalities were expected to occur in the KD treatments lasting longer than 10 minutes. The anesthetic manufacturer's recommendation and use instructions indicated that there is a relationship between mortality and extended KDs as well (Argent Chemical Laboratories 2011). In addition, mortality is an expected outcome when fish are held in anesthetic for long periods and/or at high doses, and/or when they are exposed to handling stress (Strange and Schreck 1978; Carter et al. 2011). In a related study, no mortalities occurred when YCH were held in 25 mg L^{-1} of MS-222 for 180 minutes; yet the dose of 100 mg L^{-1} of MS-222 was lethal at 30 minutes (Strange and Schreck 1978). With 30 minutes of handling stress at 50 mg L^{-1} of MS-222, YCH experienced mortalities at a rate lower than the 100 mg L^{-1} of MS-222 at 30 minutes (Strange and Schreck 1978). In a related species, rainbow trout (*O. mykiss*), fingerlings could be held at 60 mg L^{-1} MS-222 for 15-minute exposures with no mortality but 15 minutes of exposure at 80 mg L^{-1} led to 80% fingerling mortality (Gilderhus and Marking 1987). Thus, dose, KD, fish condition, and species are relevant when assessing the possibility of fish mortality related to anesthetic stress.

Several factors may have influenced the expected mortality experimental outcome in this study. First, juvenile YCH were likely exposed to low doses of MS-222 during handling stresses (i.e., tank changes or length-weight measurements) and to therapeutics for disease management prior to their inclusion in this study. Hatchery-related exposures like this could have influenced the experimental outcome by increasing the resilience, and thus stress-tolerant responses of the YCH. Woodward and Strange (1987) found that hatchery-reared rainbow trout had a dampened stress response (i.e., reduced cortisol concentration) when compared to wild rainbow trout. Hatchery rainbow trout when compared to their wild counterparts also recovered more quickly from the stress treatments (0.5 days for hatchery rainbow trout and 4 days for wild rainbow trout). Second, the surgeons in this experiment were trained to control the anesthetic and maintain the fish in Stage 4 sedation by using water and MS-222. It is possible that the

action of flushing of a lower dose or freshwater across the gills was enough to bias our results. Lastly, during the spring and summer when many of the Columbia River telemetry studies occur, the outmigrating subyearling and yearling Chinook are in a heightened physiological state of smoltification. Fish used in a telemetry study go through additional stressors, not tested in this study, such as moving into and holding in dam systems, being exposed to an anesthetic for telemetry selection from the run-of-the-river fish, and then undergoing anesthetization and handling again for the telemetry tagging. Mortalities may occur more often than expected in the field, but field conditions were poorly demonstrated in this study, because the YCH were not smolting or advancing towards smolting, had less anthropogenic stress, and were not repeatedly exposed to MS-222.

Using ice water to induce a physical state where blood sampling of the YCH could be conducted without the confounding effect of MS-222 was effective for sampling. However, the anesthetic control YCH clearly indicated a state of osmotic stress where the sodium and calcium concentrations were significantly greater than most KD treatments on day 0. Physiologically, nerve conduction is negatively correlated with temperature, for example as temperature decreases, nerve conduction decreases, suggesting that exposure to cold temperatures can act as an anesthetic and slow metabolism and the hypothalamic-pituitary-interrenal axis response. Thus, one would have expected the YCH in the anesthetic control group to have lower cortisol levels in the experiment regardless of KD or day of treatment. While the AC group's mean cortisol level was lower than fish surgically implanted with tags, there was little difference between AC and SC groups, which were exposed to MS-222.

MS-222 minimized the effects of handling and invasive procedures (e.g., surgeries). These results showed the degree of CNS depression was effective in modulating some physiological responses, such as plasma cortisol (Cho and Heath 2000; Congleton and Wagner 2006). For example, the Surgery Indicator of cortisol on days 1, 7, and 14 for the SC treatment was significantly lower than all other KD treatments, indicating that the MS-222 decreased the effects of handling; however, the fish that were exposed to surgeries had greater cortisol concentrations. The SC YCH had a significant decrease of sodium and calcium between day 1 and day 14, with no change in blood pH. Blood pH declined and plasma cortisol increased in all surgically implanted fish between day 1 and day 14, indicating recovery from the surgery and/or tag presence. The increase in plasma cortisol concentrations indicated that the anesthetic dampened the stress response (Spotte et al. 1991). Thus, surgically implanted YCH exhibited a higher overall stress response than those not surgically implanted, suggesting that surgery itself may mask the effects of the anesthetics when looking at short-term KD intervals of 5 minutes or less.

Several events may have confounded the KD exposure cortisol results. Based on the literature, we expected that the anesthetization, at MS-222 $\geq$100 mg L^{-1}, plasma cortisol levels would have been mitigated, and not produce immediate changes (Strange and Schreck 1978; Barton et al. 1986; Cho and Heath 2000). However, we anesthetized fish at 80 mg L^{-1} during the experiment, and then were required to subject them to a second dose of MS-222 at 250 mg L^{-1} to retrieve blood samples from fish sampled on days 0, 7, and 14. This second exposure of MS-222, although short in duration, may have allowed for elevated plasma cortisol compared to the initial response of long exposures needed to implant the transmitters. Because the plasma cortisol levels were elevated in all KD groups on all days, this may have been an incorrect assumption. Lastly, the study was conducted in early spring. It is possible that the increase in cortisol levels could also be affected by the change in photoperiod and initial stages of smolitifcation (McCormick et al. 2002; Björnsson et al 2011; Steffansson et al. 2009). Thus, plasma cortisol may not be an effective measure of tagged fish condition as they migrate through the FCRPS.

The blood analyte levels were included in this study as an indicators of osmoregulatory imbalance and secondary stress response to the anesthetic and surgery that may also be indicative of sub-lethal stress, delayed mortality, and mortality rate (Järvi 1990; Barton and Iwama 1991; Wendelaar Bonga 2011). On day 0, there was an indication that YCH held between 6 and 9 minutes past anesthetic Stage 4 exhibited a significantly different osmotic imbalance in sodium, calcium, and potassium than YCH in the 0-, 3-, 5-, 12- and 15-minute exposures. The sodium and potassium values of the 6- and 9-minute exposures were similar to the sodium and potassium concentrations reported for YCH exposed to 100 mg L^{-1} MS-222 for 8 minutes (sodium 160 mmol L^{-1}; 2.79 mmol L^{-1}; Congleton 2006). However, the calcium concentrations are markedly lower than values reported by Congleton (2006) and Wagner and Congleton (2004), which we attributed to the calculated adjustment of calcium for pH made by the instrument, which did not occur in the values reported for calcium in the above-cited studies.

Based on the data and predictive models, the blood analytes generally moderated over the post-implantation days, indicating the YCH regained their osmotic abilities. Osmotic perturbation was likely a secondary response to the handling, anesthetic, and surgical stresses, which have been observed in handling and integument studies (Gadomski et al. 1994; Congleton 2006; Zydlewski 2010). In addition to the stressor of interest (MS-222), handling the fish and surgery itself can induce osmotic imbalance. Handling fish can affect the fish's skin permeability and thus its osmotic homeostasis (Kuperman et al. 2001; Zydlewski et al. 2010). This likely occurs during invasive surgeries were the skin is cut in order to intra-coelomically implant acoustic tags. If the incised skin is not apposed well or tension across the wound does not keep the incision closed, the *internal milieu* of the fish is compromised by the exchange of fluids between the peritoneal cavity and the aquatic environment. During sampling on days 7 and 14, there were no open or poorly healing incisions noted on the study fish. There were no differences in the analytes between day 7 and day 14, possibly indicating that the stress of MS-222 and surgical implantation were less; however, we cannot determine from the results whether this was related to incision healing.

This study was conducted to address the CBSPSC concerns that a 10-minute exposure to 80 mg L^{-1} MS-222 may affect the downstream survival of outmigrating YCH. Based on the results of this study, it is recommended that the "10-minute maximum exposure to MS-222" suggested by the committee remain in place until further research indicates KD points or procedures that can mitigate or reduce the overall stress response of juvenile YCH to the surgical implantation process. The results of this study did not strongly indicate that one KD period was more or less stressful than another. In addition, there were no mortalities observed in any control group or surgically implanted YCH. It was apparent that surgically implanted YCH exhibit an overall higher stress response, including analytes and biochemistry data, than those not surgically implanted, suggesting that surgery itself may mask the effects of the anesthetics. There were indications that the fish experienced "normal" anesthetic stress, as on day 0, and subsequent changes in physiological state as indicated by the blood analyte and biochemistry concentrations, which generally reduced by day 14. In addition, the KD treatments of 6 and 9 minutes tended to yield results different from other KD treatments regardless of the post-implantation monitoring day. Researchers are cautioned to remember that many factors may have affected the stress responses measured in this study, including anesthetic dose (Smit and Hattingh 1979), water hardness (Gilerhus et al. 1973), temperature (Carter et al. 2011), fish size and past exposure history (Oikawa et al. 1994; Bystrainsky et al. 2002), and study sample size.

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