The acutely lethal toxicity of urban stormwater runoff to juvenile coho salmon

Michelle I. Chow

A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2018

Committee:

Graham Young

Jenifer K. McIntyre

Nathaniel L. Scholz

Steven B. Roberts

Program Authorized to Offer Degree:

Aquatic and Fishery Sciences

University of Washington

Abstract

The acutely lethal toxicity of urban stormwater runoff to juvenile coho salmon

Michelle I. Chow

Chair of the Supervisory Committee:
Dr. Graham Young
School of Aquatic and Fishery Sciences

Stormwater runoff is considered the leading source of non-point source pollution in watersheds under pressure from urban development. In urban creeks around Puget Sound, WA, several lines of evidence indicate that toxic urban runoff is responsible for high rates of premature mortality among adult coho salmon (*Oncorhynchus kisutch*) that return from the ocean in the rainy fall months to spawn. The current study is a detailed examination of the behavioral and physiological aspects of the urban mortality syndrome in juvenile coho. Juveniles were exposed to either clean (control) hatchery water or stormwater collected from a high traffic volume arterial roadway. Runoff-exposed fish showed the same progression of symptoms (distress) as previously documented for adult spawners in past field surveys. This behavioral sequence was characterized in detail, and discrete stages of distress were then used as phenotypic anchors for physiological analyses of blood and tissue chemistry along a gradient of symptomology, from outwardly normal (presymptomatic) to a several loss of equilibrium (near death). These analyses showed that

urban stormwater exposures caused a dysregulation of blood ion and osmoregulatory homeostasis in juvenile coho salmon, as previously observed in adults. The commonalities across life stages suggest that juveniles can provide an experimental platform for identifying the chemical agents and toxicological mechanisms underlying the mortality syndrome in adults. This is an increasingly important conservation challenge for wild coho populations throughout rapidly urbanizing regions of the Pacific Northwest.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I first want to acknowledge Environmental Protection Agency, Region 10 for funding, as this research would not have been possible without it. I would also like to thank the scientists and collaborators of the Puget Sound Stormwater Science Team for their contribution and dedication to stormwater science. I would like to specifically thank Jay Davis for managing funding, Jessica Lundin for her invaluable help on statistical techniques, David Baldwin for help with experimental design, Emma Mudrock for her technical assistance, Louisa Harding for general guidance, Jill Wetzle for her support, and James Cameron for his technical knowledge. Additional support for experiments came from Ken King, Chelsea Mitchell, Ben Leonard, Erica Brown, and Cathy Laetz. Special guidance and assistance on laboratory procedures were from Jasmine Prat, Mollie Middleton, and Brian Beckman. I would also like to thank the entire Ecotoxicology Program at the Northwest Fisheries Science Center, Washington State University Puyallup, and my fellow students and professors at University of Washington in the School Aquatic and Fishery Sciences. Without the collaborative effort and support between these institutions, this project would not have been possible. Most of all though I would like to thank Graham Young for his guidance and encouragement, Jenifer McIntyre for her mentorship, Nathaniel Scholz for his perspective and leadership, and lastly Steven Roberts for his support.

Chapter 1. INTRODUCTION

As the global human population increases, the concomitant expansion of cities and extended metropolitan areas is profoundly changing the structure and function of freshwater ecosystems. This phenomenon is known as the urban stream syndrome (Paul and Meyer 2001; Meyer et al. 2005; Walsh et al. 2005), wherein river networks experience a flashier hydrograph, altered channelization, increased levels of contaminants and nutrients, and reduced biotic richness (Paul and Meyer 2001; Meyer et al. 2005). These and related forms of habitat degradation are primarily attributable to landscape-scale changes in land use.

In coastal regions of western North America, urban growth has been relatively rapid in recent decades. This includes, for example, lowland areas throughout the Puget Sound Basin (greater Seattle metropolitan area) as well as the Lower Columbia River Basin (greater Portland metropolitan area). Around Puget Sound, nearly 25% of the total land area is currently considered developed (Bolte and Vache 2010), and the region is anticipating another 1.5 million people in the coming decades (Puget Sound Regional Council 2009). Associated with ongoing development is the important forcing pressure on land cover change. This often includes a loss of historical forests within watersheds and an increase in imperviousness involving the expansion of compact soils, roadways, parking lots, buildings, and other structures.

In the Pacific Northwest, the negative influences of urbanization on the physical processes that shape aquatic habitats have been studied for decades (Booth et al. 2002). The consequent increase in stormwater runoff (surface water volume) mobilized by impervious surfaces changes the hydrology of stream and river systems. This includes flashier flows (streambed scour), bank erosion, sedimentation, and other processes that are known to impact stream communities. Past studies have primarily focused on macroinvertebrate abundance and diversity as *in situ* indicators of biological integrity in streams known to be hydrologically altered by urbanization (e.g., Morley and Karr 2002; Alberti et al. 2007). This is, in part, the basis for the so-called “10 percent rule”, whereby degradation within a watershed is evident when effective impervious area exceeds 10% (Booth and Jackson 1997). Although less studied, physical habitat changes associated with urbanization have also been associated with declines of

Pacific salmon, including Chinook (Moscrip and Montgomery 1997; Regetz 2003) and coho (Pess et al. 2002; Bilby and Mollot 2008).

Given the demonstrated vulnerabilities of urban streams to changing flow regimes, mitigation and restoration efforts have worked to reduce the volume of stormwater runoff from impervious areas (Booth et al. 2004). For example, since at least the early 1980s, King County in central Puget Sound has been implementing structural and non-structural strategies to reduce the cumulative impacts of urbanization (Booth et al. 2002). More generally, however, habitat restoration to promote salmon recovery in the Pacific Northwest has overwhelmingly focused on physical habitat improvements (Katz et al. 2007). By comparison, the role of water pollution in salmon declines has received much less attention, even in urban watersheds where water and sediment quality may be particularly poor. There are several reasons for this information gap, including the cost and logistical difficulty of measuring non-conventional water quality parameters – e.g., toxic chemical contaminants – in dynamic, free-flowing aquatic systems. Moreover, Pacific salmon have highly migratory life histories, and thus may integrate the health impacts of contaminant exposures over large geographic ranges (Ross et al. 2013).

In the Pacific Northwest and elsewhere throughout North America, stormwater runoff is the primary driver for non-point source pollution in urban watersheds (Watkins et al. 2004). Rain falling on impervious surfaces mobilizes complex mixtures of contaminants, which are then transported and discharged to surface water habitats. For a given storm event, the amount of pollution carried in runoff may vary as a function of seasonality, rainfall timing and intensity, drainage area and other factors. However, stormwater collected from or near roadways with dense traffic volumes has long been known to be particularly toxic to aquatic species (Marsalek et al. 1999). Cars and trucks release a very large diversity of chemicals into the environment as a consequence of tire and brake pad wear, the leakage of crankcase oil and transmission fluid, and tailpipe exhaust. These contaminants, many of which have not been previously studied, accumulate on roadways and other impervious surfaces. For example, a recent assessment of Seattle-area highway runoff using high-resolution mass spectrometry has revealed hundreds of distinct chemicals with unknown identities and unknown impacts on the health of fish and other aquatic species (Du et al. 2017).

In Puget Sound, conventional (i.e., untreated) urban runoff is demonstrably toxic to fish and macroinvertebrates, including juvenile salmon and their prey species. Adverse health effects

range from developmental abnormalities in fish embryos (McIntyre et al., 2014, 2016) and reproductive failure in cladocerans (McIntyre et al. 2015) to acute lethality in mayflies and juvenile coho salmon (McIntyre et al. 2015). Over the past two decades, however, considerable attention has been focused on the health and survival of adult coho that return from the ocean each fall to spawn in lowland urban streams. Beginning in the late 1990s, field surveys were implemented in small Seattle-area watersheds to evaluate the effectiveness of stream restoration projects (e.g., culvert removals, invasive species removal, etc.). This led to the discovery of an urban stream mortality syndrome, characterized by the pre-spawn death of gravid coho salmon females. Overall die-off rates have been very high – up to 90% of the fall run for a given watershed (Scholz et al. 2011). Prior to dying, affected fish show a consistent progression of behavioral symptoms including a loss of orientation, circular surface swimming and gaping, a loss of equilibrium, and immobility. These symptoms have been consistently observed across many years and several urban watersheds, suggesting a consistent cause of death. This may involve a severe metabolic acidosis and ion regulatory disruption, as evidenced by a recent study that examined the blood chemistry of overtly symptomatic adults exposed to highway runoff (McIntyre et al. 2018).

Several lines of evidence suggest that toxic stormwater runoff is killing coho. First, forensic analyses have largely ruled out conventional water quality parameters, spawner condition, disease, and other alternate hypotheses (Scholz et al. 2011). Second, the mortality syndrome, including symptoms, can be reproduced in experiments wherein adult coho are exposed to highway runoff under controlled conditions (Spromberg et al. 2016). Finally, landscape modeling has shown that the severity of coho spawner mortality scales with the extent of imperviousness within a watershed (Feist et al. 2011) and, more specifically, the density of motor vehicle traffic (Feist et al. 2017).

Given that coho salmon only spawn before dying (semelparity), the loss of large numbers of fish at the terminal (spawner) life stage can have disproportionately negative consequences for wild populations. In terms of current conservation status, the Puget Sound coho population segment is a species of concern under the U.S. Endangered Species Act. In the Lower Columbia River, the species status is threatened. As expected, initial modeling suggests that future urbanization, increased toxic runoff, and increased spawner mortality has the potential to drive rapid local population extinctions (Spromberg and Scholz 2011). Retrospectively, this may

partially explain declining abundances of coho in urbanizing watersheds, as determined by field assessments (Pess et al. 2002; Bilby and Mollot 2008).

Notably, urban runoff can be expected to further limit wild coho populations if other life stages are impacted. In this context, this Chapter is focused on the vulnerability of coho juveniles. Encounter rates for juveniles are relatively low during fall surveys for adult coho spawners in Puget Sound urban streams. However, following storm events, dead juveniles have been observed in the field, as well as juveniles in obvious physiological distress, as indicated by disoriented surface swimming (N.L. Scholz, unpublished observations). Moreover, as with adults, direct exposures to highway runoff are lethal to juveniles, and this is prevented by pre-treatment with soil bioinfiltration (McIntyre et al. 2015). This suggests that the underlying causes of mortality in juveniles and adults may be similar or even the same. If so, juveniles might offer a much more tractable experimental platform for studying the syndrome, given that adults are logistically challenging to work with, unpredictable in their return rates, and limited to a few weeks each year in terms of availability. Studies on juveniles may also shed light on why coho appear to be much more vulnerable to the mortality syndrome that other species of Pacific that also spawn in urban habitats (e.g., chum; McIntyre et al. 2018).

The primary goal of this Chapter was a detailed examination of the urban runoff mortality syndrome in juvenile coho. Video analyses and related methods were used to monitor the temporal progression of behavioral symptoms in stormwater-exposed juveniles relative to control fish in clean water. These observations yielded distinct categories of distress that corresponded very closely to previous field observations of affected adults and juveniles in urban streams. Three categories of severity were then chosen as phenotypic anchors for physiological analyses. These included presymptomatic (normal behavior), intermediate distress (surface), and severe distress (loss of equilibrium). Blood and tissue samples were then collected from coho in each of these symptomology categories and analyzed for a range of blood chemistry and histological parameters, including blood gases and ions, red blood cell indices, and conventional enzyme assays. These metrics were used to evaluate deviations away from normal homeostasis in runoff-exposed fish. Results are interpreted in the context of likely toxic mechanisms and target tissues involved in the mortality syndrome.

Chapter 2. THE BEHAVIORAL AND PHYSIOLOGICAL CONSEQUENCES OF URBAN STORMWATER RUNOFF TO JUVENILE COHO SALMON

2.1 METHODS

2.1.1 *Urban (highway) runoff collection*

Urban stormwater runoff was collected from downspouts draining an elevated urban principal arterial, specifically, the westbound onramp to State Route 520 from Montlake Boulevard in Seattle, WA. The ramp receives approximately 15,000 average daily vehicle trips (ADTs) and is paved with Portland cement concrete, a conventional urban impervious surface material.

During rain events, the highway runoff was pre-filtered through a fiberglass screen to remove coarse debris and then collected in a 900-L stainless steel tote (Custom Metalcraft Inc., Springfield, MO). The stormwater was subsequently transported to Washington State University Puyallup Research and Extension Center (WSU-Puyallup; Puyallup, WA) and used for juvenile salmon exposures within 24 hrs post-collection. Samples for analytical chemistry were also collected within 24 hrs, prior to experimental exposures (Section 2.1.6).

2.1.2 *Juvenile coho salmon*

Juvenile coho salmon (aged 1+ year) were obtained from the Northwest Fisheries Science Center hatchery facility (Seattle, WA) and maintained at WSU-Puyallup in circular fiberglass tanks supplied with dechlorinated municipal water at 13 °C. Fish were held on a 12:12 light:dark regime and fed daily with commercial pellet food (Bio-Oregon, Warrenton, OR). At the time of the behavioral trials, fish averaged (mean ± SD) 53.4 ± 28.7 g in weight and 162.9 ± 30.8 mm in length. Fish exposed for physiological analyses, approximately two months later, averaged (mean ± SD) 208.4 ± 98.2 g in weight and 255.6 ± 43.6 mm in length.

Exposed coho salmon were euthanized by blunt force to the head when fish reached a moribund state unless otherwise specified. “Moribund” describes salmon that were immobile on the bottom of the exposure tank and were unresponsive to touch. Experiments were conducted in

accordance with Experimental Protocol #04860-002, as approved by Washington State University's Institutional Animal Care and Use Committee (IACUC).

2.1.3 *Synthetic stormwater exposures*

Relative to clean hatchery water, urban runoff contains mixtures of salts that could potentially affect the osmolality of exposed coho salmon, independent of toxic chemical contaminants. Therefore, to eliminate the possibility that changes in conventional water quality parameters (e.g., pH, alkalinity, hardness) could alone induce changes in the blood chemistry of exposed fish, synthetic stormwater was created based on stormwater chemistry values collected between 2012-2013 (Spromberg et al. 2016; Table A.1).

Synthetic stormwater was created to approximate the conventional chemistry of the control hatchery water, as well as low and intermediate salt concentrations previously measured in runoff from the State Route 520 onramp collection location (see above). Synthetic stormwaters were constituted by filtering deionized water through a carbon filter (Brita LP, Oakland, California) and a Synergy water purification system (EMD Millipore, Darmstadt, Germany) and then adding salts ($CaCl_2 \bullet H_20$, $Na \bullet HCO_3$, $MgCl_2 \bullet 6H_20$, and KCl). The pH of the synthetic waters was adjusted using HCl and NaOH as appropriate.

Five juvenile coho salmon were exposed to each of four treatments (control hatchery water; synthetic control hatchery water; synthetic "low" stormwater; synthetic "intermediate" stormwater) in 35-L glass aquaria supplied with air stones. Temperature was maintained at 13 °C by placing aquaria in flow-through circular fiberglass tanks. Beginning at 4.5 hrs after the onset of each exposure, fish were sampled for arterial blood. Collected blood was analyzed on the iSTAT point-of-care blood analyzer using the methods outlined below.

2.1.4 *Behavioral characterization*

Experiments to assess behavioral symptomology among runoff-exposed juvenile coho were conducted in twelve 56.6-L acrylic tanks (61 cm x 46 cm x 37 cm) in an isolated wet laboratory (Figure A.1). Whereas the sides of the tanks were opaque, the bottoms used clear plexiglass. The tanks were elevated on steel stands to allow observations from below. Urban runoff was collected from two separate rainfall events on April 10 and 23, 2017. For each storm,

exposures were conducted on two consecutive days. Runoff to be used on the second day was stored overnight in the stainless steel transport tote at ambient room temperature (18 °C).

Six of the acrylic tanks were filled with clean hatchery (control) water and maintained under static conditions with air stones to provide supplementary oxygenation. The remaining six tanks, in two rows of three, were plumbed to supply recirculating urban runoff or control water with flow rates set to 6 L/min. Dissolved oxygen, temperature, pH, and conductivity were monitored throughout each of the recirculating exposures.

In pilot trials to assess handling stress (data not shown), a 2 hr acclimation period was sufficient for juvenile coho to return to normal behavior following an initial transfer between tanks and then a subsequent transfer to a third tank. Therefore, on a given day, six pairs of juvenile coho were transferred from the rearing tanks to each of the six acrylic holding tanks (static clean water), allowed to acclimate for 2 hr, and then each pair was moved by net to one of the six recirculating exposure tanks at the outset of the experiment (Figure A.2).

Departures from normal swimming behaviors were quantified from video collected using three overhead cameras (Dahua Technology Lite Series 1.3MP; Irvine, CA) connected to SecuritySpy video capture software (Ben Software, London, United Kingdom). Each camera was positioned to monitor four tanks, allowing all tanks to be simultaneously observed. However, due to the opacity of the stormwater, the cameras only captured behaviors near the surface of each tank in runoff-exposed fish. Accordingly, control behaviors were only quantified when the fish were near the surface of the water. Moribund fish at the bottom of the tank were documented manually by visual observations through the plexiglass.

On each experimental day, for each of the six recirculating exposure tanks, the pair of fish was divided into two parallel experiments (Figure A.2). In the first, one of the fish remained in each tank to monitor the entire progression from normal behavior to more severe symptomology deviating from control behavior. The goal of the second experiment was to determine whether symptomatic coho would recover upon transfer to clean water. Accordingly, the other fish in the stormwater-exposed pair was moved back into a static clean water tank at a point of intermediate distress (surface swimming). At that time point, a corresponding control fish was also moved from the recirculating tank back into a clean water static tank. Runoff-exposed fish transferred to clean water were observed until they reached a moribund state. To control for the additional transfer stress, the remaining fish in each exposure tank was also

dewatered for a few seconds. All exposures were terminated after eight hours. Video recordings were analyzed using Timestamped Field Notes (Neukadye, Golden, CO).

Overall, for the categorization of symptomology, a set of three stormwater-exposed and three control fish were evaluated each day, over two days, for an n = 6 for each treatment per storm. The behavioral progression of the runoff mortality syndrome (Table 2.1) was used to define distinct stages of symptomology, ranging from Stage 0 (presymptomatic) to Stage 6 (moribund). Subsequent collections of blood and tissues were sampled at Stage 0,3, and 4 (see below).

2.1.5 *Physiological characterization*

2.1.5.1 Runoff exposures

Direct exposures to urban runoff have previously been shown to dramatically alter a range of hematology parameters in adult coho, including ion balance and blood gases (McIntyre et al. 2018). A similar blood chemistry assessment was conducted here, primarily based on analyses of arterial blood from juvenile coho using a point-of-care blood analyzer (iSTAT; Abaxis, Union City, CA).

Juvenile coho salmon were exposed to urban runoff collected from a storm event on June 8, 2017 (n = 45 fish), or hatchery control water (n = 45 fish) at WSU-Puyallup. Exposures were conducted in the twelve acrylic tanks described above after they were re-plumbed to supply recirculating storm- and clean hatchery water to a row of six tanks for each. Flow rates were set to 6 L/min. Dissolved oxygen, temperature, pH, and conductivity were monitored throughout the exposure.

Runoff-exposed fish were observed along the consistent time course of symptomology and then sampled at discrete points (Stages) in the syndrome, as determined from the behavioral assessments above (see also Table 1). The three discrete behavioral categories included Stage 0 (presymptomatic, or normal swimming behavior), Stage 3 (surface swimming), and Stage 4 (loss of equilibrium). The elapsed time to reach each Stage (mean ± SD) was 62.2 ± 18.3 min (Stage 0), 125.1 ± 49.5 min (Stage 3), and 199.6 ± 63.2 min (Stage 4).

Six tanks, each containing five fish, were used for each Stage (n = 15 fish per treatment). The start of each exposure was staggered to allow adequate sampling time. Behaviors were closely monitored using the overhead camera system described previously. When a runoff-

exposed fish reached the targeted behavioral Stage, it was removed from the tank and euthanized by blunt force to the head. A corresponding control fish from the paired clean water tank was then sampled.

2.1.5.2 Blood chemistry

Immediately after euthanizing an individual fish, up to 1 mL of arterial blood was collected from the dorsal aorta using a 25-gauge, 1-in needle connected to a lithium-heparinized syringe (7 IU/mL ion-balanced lyophilized; ABG Line Draw, Smiths Medical, Dublin, OH). After brief mixing by horizontally rolling the syringe, a drop of blood was discarded and 95 µL of blood was loaded into a CG8+ iSTAT System Test Cartridge (Abaxis, Union City, CA). The cartridge was then inserted into an iSTAT point-of-care blood analyzer (Abaxis, Union City, CA) for analysis. Blood metrics analyzed included sodium (Na^+), potassium (K^+), ionized calcium (iCa^{2+}), partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2), pH, glucose (GLU), and hematocrit (HCT). Other parameter outputs from the iSTAT, including bicarbonate (HCO_3^-), total carbon dioxide (TCO_2), oxygen saturation (sO_2), extracellular base excess, and hemoglobin, were indirectly calculated and therefore not included in subsequent analyses. Although hematocrit is consistently underestimated on the iSTAT by 8-10% (McIntyre et al. 2018; Harrenstien et al. 2005), hematocrit values are reported as measured.

Following previously described methods (Abbott 2016, Abbott 2013a, Abbott 2013b; Gallagher et al. 2010), temperature corrections were applied for temperature-sensitive parameters (pH and gases) using the average temperature of the exposure water (Table A.2):

Equation 2.1:

$$pH_{TC} = pH_M - 0.014(T - 37) + 0.0065(7.4 - pH_M)(T - 37)$$

Equation 2.2:

$$pCO_{2,TC} = pCO_{2,M}\left(10^{0.019(T-37)}\right)$$

Equation 2.3:

$$pO_{2,TC} = pO_{2,M} \times 10^{\frac{5.49\times10^{-11}\,pO_{2,M}^{3.88}+0.071}{9.72\times10^{-9}pO_{2,M}^{3.88}+2.30}(T-37)}$$

T: exposure water temperature

M: measured value on the iSTAT

TC: temperature corrected value

When the iSTAT analysis was complete, a 50 µL aliquot of whole blood was collected and stored at 4 °C for quantification of red blood cell indices. The remaining whole blood of each sample was centrifuged for 5 min at 12,000 x *g* to separate plasma and red blood cell pellets, followed by storage at -80 °C.

Red blood cell indices were determined for fish sampled at Stage 4 (loss of equilibrium) within 24 hrs of blood collection. Briefly, 10 µL of blood was diluted 200-fold with saline (2.4 mM $CaCl_2$, 4.3 mM $MgCl_2$, 10 mM KCl, 140 mM NaCl) and Trypan Blue (0.4%). The blood solution was then loaded in 10 µL volumes into each side of a Double Neubauer Hemocytometer (Hausser Scientific, Horsham, PA). Viable and non-viable cells were quantified across five 0.04-mm^2 squares on each side of the hemocytometer. Cells that were permeable to Trypan Blue, as indicated by blue cytoplasm, were characterized as non-viable (Strober 2001). If duplicate readings of a sample were significantly different (t-test, $\alpha = 0.05$), the sample was reanalyzed. Total red blood cell counts were adjusted for dilution and reported as 10^{12} cells/L of whole blood. Mean corpuscular volume of the blood cells (MCV) was calculated as follows:

Equation 2.4:

$$MCV = \frac{HCT}{Total\ RBC \times 10^{-1}}$$

MCV: Mean corpuscular volume (femtoliters, fL)

HCT: Measured hematocrit, percent packed cells in whole blood measured on the iSTAT

Plasma lactate and total protein were measured on a VetTest Chemistry Analyzer (IDDEXX Laboratories, Westbrook, ME). After thawing on ice, a 50 µL aliquot of plasma was warmed to room temperature and diluted 1:1 with saline (154 mM NaCl, pH 7.0) for plasma analysis; the remaining plasma was refrozen. Within a few days, plasma was rethawed and

quantified from duplicate 10 µL samples on a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, Utah).

2.1.5.3 Tissue analyses

Fish were measured for length and weight immediately after blood sampling. Condition factor was calculated using Fulton's Condition Factor (Ricker 1975):

Equation 2.5:

$$Condition\ Factor\ (K) = \frac{weight\ (g)}{length\ (cm)^3}\ x\ 100$$

Conventional enzyme assays were used to evaluate the function of ion transporters in posterior kidneys and gills using previously described methods (Schrock et al. 1994; Chowdhury and Wood 2016). For Na^+, K^+ and H^+-ATPase activity assays, sections of tail kidneys and soft gill tissues (dissected from the cartilage, < 5 mm) were collected from each fish, rinsed with SEI buffer (250 mM sucrose, 50 mM imidazole, 10 mM Na2EDTA, pH 7.3), and stored in buffer at -80 °C.

Within three months, conventional enzyme assays were conducted. Four unique assay mixes were prepared daily. The first (*A*), a negative control, contained no enzyme inhibitor. The second (*B*) included 0.03 mM ouabain to block Na^+, K^+-ATPase activity. The third (*C*) contained both 0.03 mM ouabain and 500 mM sodium azide to inhibit Na^+, K^+-ATPase and background mitochondrial ATPase activity. The final mix (*D*) added 1 mM N-ethylmaledimide to *C* to additionally block V-type H^+-ATPase activity. All assay mixes were constituted from a working stock containing 2.8 mM phosphoenol pyruvate (PEP), 3.5 mM ATP, 0.2 NADH, 4U/mL lactate dehydrogenase (LDH), and 5U/mL pyruvate kinase (PK).

To measure the rate of ATPase hydrolysis, tail kidney and gill tissues were homogenized on ice in SEID buffer (SEI buffer plus 0.3% sodium deoxycholic acid) and centrifuged for 3 min (5,000 x g, 4 °C). The supernatant was transferred to a clean tube and 10 µL of each sample was added in duplicate to a 96-well round bottom plate on ice. The remaining homogenate was stored at -20 °C for protein quantification. Each assay mix (*A* - *D*) was combined with a salt solution (189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, 50 mM imidazole, pH 7.5) at a 3:1 ratio, and then

200 µL was added to each well. Absorbance was measured at 340 nm for 10 minutes at 25 °C using a microplate reader (VersaMax, Molecular Devices LLC, Sunnyvale, CA).

To standardize the ATPase hydrolysis measurements, the protein content of each homogenate was measured (Schrock et al. 1994). To this end, homogenates were diluted 1:1 with BSA (0.2% bovine serum albumin) and 4 µL of the dilution was added in duplicate to a 96-well flat bottom plate. Subsequently, 70 µL of protein mix ($CuSO_4$, Na_2CO_3, and Na-K tartrate solution) was added and plates were placed on an orbital shaker for 5 min. Folin's reagent (210 µL) was added to each well and the plate was shaken for another minute, incubated at 37 °C for 5 min, and finally incubated for 30 min in the dark. At the termination of the reaction, plates were read at 550 nm.

Rates of Na^+, K^+-ATPase activities were determined by subtracting the background ATP hydrolysis rate in the control mix *A* and the ouabain-containing mix (*B*) and then normalizing to the protein content of each sample (*A*-*B*/[protein]). Rates of V-Type H^+-ATPase were similarly determined by subtracting absorbance of assay mix *D* from assay mix *C* before normalizing by the protein concentration of the homogenate (*C*-*D*/[protein]).

2.1.6 *Analytical chemistry*

Exposure water samples (hatchery control water and all three storms) were collected prior to the onset of juvenile coho exposure trials and analyzed for conventional water quality parameters by a commercial laboratory (Analytical Resources Inc., Tukwila, WA) using US EPA approved methods. Targeted parameters included ammonia, total suspended solids, and dissolved organic carbon. In addition, total and dissolved concentrations of arsenic, cadmium, copper, lead, nickel, and zinc were measured by the same laboratory using inductively coupled plasma mass spectrometry (ICP-MS).

2.1.7 *Statistical analyses*

For univariate analyses, data were screened for normality using kurtosis and skewness (timeDate R package; Wuertz et al. 2015). Equal variance assumptions were tested using the Bartlett's test. Outliers greater than three standard deviations from the mean for each treatment were excluded. Log transformations were applied for Na^+, $iCa^{2+,}$ glucose, hematocrit, and lactate to meet normality and equal variance assumptions required by analysis of variance (ANOVA).

Values for total protein were not transformed given that none of the applied transformations met the required assumption of equal variance.

Two-way ANOVAs were used to compare between treatments and behavioral symptomology (Stage) for all measured parameters including behaviors, blood metrics, and tissue analyses. Post-hoc analyses were conducted using Tukey's Honest Significant Difference test to determine the significance of pair-wise differences. Across sampling Stages, replicate tanks were analyzed together since blood metrics were consistent between replicates.

For measured blood metrics, principal component analyses (PCA) were also applied to identify specific blood profiles associated with each treatment and symptomology Stage. PCA is a linear ordination technique that uses Euclidean distances to describe correlations among measured variables. This reduces the dimensionality of the multivariate matrix into synthetic dimensions (principal components) for easier data interpretation. Measured blood metrics (n = 11) were pre-screened for multivariate outliers and individual fish for which some data were missing. Three fish were removed from analysis given a Euclidean distance standard deviation greater than three. Fourteen additional fish were omitted due to missing blood metric data. In total, six PCAs were conducted (vegan R package; Oksanen et al. 2015). Analyses were conducted on datasets separated by treatment ($n_{control} = 33$, $n_{runoff} = 33$) and by symptomology Stage ($n_{Stage\ 0} = 25$, $n_{Stage\ 3} = 21$, $n_{Stage\ 4} = 20$).

For each dataset, PCA was conducted on a correlation matrix to standardize among variables (mean = 0 and standard deviation = 1). The significance of eigenvalues was tested using the Broken Stick Model and a Monte Carlo randomization test with 1000 permutations. Both tests determine if derived eigenvalues diverge from those predicted by a null hypothesis. Principal components with significant eigenvalues and high variable loadings (> 0.25, absolute value) are considered important for explaining the observed variance and were therefore retained. Eigenvectors were varimax rotated and ordination biplots constructed using the scores of significant principal components. To statistically test if fish loaded differently along principal components depending on behavioral Stage or treatment, linear regression models were applied on the principal component scores (i.e., coordinates of the biplot) for each PCA.

All statistical analyses were conducted in R (version 3.3.2, R Core Team 2016). Significance was set at $\alpha = 0.05$ in all cases.

2.2 RESULTS

2.2.1 *Synthetic stormwater does not induce the urban mortality effect*

Synthetic stormwater treatments did not have significant effects on behavioral symptomology or blood metrics measured in exposed coho salmon. Coho salmon exposed to synthetic stormwater exhibited none of the behavioral symptoms characteristic of fish exposed to urban runoff. Furthermore, out of the eight measured iSTAT blood variables, only iCa^{2+} was significantly different between treatments ($p = 0.017$). No other disturbances in blood chemistry were detected.

2.2.2 *Urban runoff exposures cause a progressively severe suite of behavioral symptoms in juvenile coho salmon*

Exposures to collected highway runoff were almost universally lethal to juvenile coho salmon, with 96% of all exposed fish (n = 23 of 24) losing equilibrium and becoming immobilized on the bottom of the exposure tanks (i.e., moribund) within seven hours (mean ± SD: 236 ± 84 min) in both transient and continuous exposures. In addition to monitoring behavior, these experiments also addressed whether symptomatic fish can recovery if returned to clean water. The answer appears to be no, as juvenile coho in an intermediate stage of distress (surfacing) continued along the progression of symptoms to the near-death terminal Stage 6 (moribund) following transfer to clean water. Relative to controls (no mortality), there was no difference in the proportion of moribund coho between those continuously exposed to runoff and those transferred to clean water during an initial phase of symptomology (Figure 2.1). The effects of stormwater were consistent, as there were no significant differences in the average time to a moribund state between transient and continuous exposures ($p = 0.371$), across the two storms ($p = 0.793$), or between the first and second day of exposures for a given storm ($p = 0.144$; Figure 2.2).

The progression of symptoms for runoff-exposed coho closely resembled the behaviors previously reported for adult coho returning to spawn in Puget Sound urban watersheds (Scholz et al. 2011). The sequence was as follows: discrete surfacing events > continuous surface swimming > short bursts of high velocity swimming > loss of equilibrium > loss of buoyancy > unresponsive (moribund). The distinctive characteristics of each of these categories, or Stages,

are described in Table 2.1. While the amount of time spent by each individual fish in a given Stage varied, all of the juvenile coho transitioned through the six categories of increasing distress.

Control fish behaved normally (no surfacing) or showed some evidence of Stage 1 behavior (brief episodic surfacing), but the latter remained relatively constant and did not progress further on the symptomology scale. In runoff exposures, there was a discrete transition phase in which runoff-exposed fish departed from the episodic surfacing behavior of the controls (Figure 2.3). Stormwater-exposed fish quickly began surfacing (Stage 1), and this soon increased in both length and frequency (Stage 2; Figure 2.3). Over time, short episodes of continuous surface swimming progressed to sustained surface swimming in large circles or around the edges of the tank (Stage 3).

As previously documented for adult spawners in the field (Scholz et al. 2011), there was individual behavioral variation at this stage. Across exposed juveniles, some swam with their snout out of the water, gaped, ran into the tank walls, or exhibited a combination of these behaviors. This was typically followed by short intervals of burst swimming, defined as short increases in propulsion that was not sustained.

Surfacing activity was sustained until the next major shift in symptomology in which fish lost equilibrium (Stage 4). In the early phase, this manifested as either a shift from a horizontal to a vertical orientation relative to the surface of the water, or the fish rolled onto their sides. Both effects on equilibrium were initially transient, but coho soon began swimming continuously on their side and/or spiraling until they eventually lost buoyancy and sank to the bottom of the exposure tank (Stage 5). Nearly dead fish on the bottom of the tank, while moribund, nevertheless continued ventilating, gaping, and occasionally exhibited spasms (Stage 6). The trial was terminated when (netted) moribund fish were no longer responsive to touch.

2.2.3 *Discrete behavioral symptoms correspond to a significant dysregulation of juvenile coho blood chemistry*

Relative to controls, stormwater exposures had the most pronounced effects on ion and osmoregulatory related blood parameters (Table 2.2; Figure 2.4). Across the three categories of increasingly severe symptomology (Stages 0, 3, and 4), the magnitude of change in Na^+, glucose, hematocrit, and osmolality concentrations varied between treatments, as indicated by an

interaction effect determined by two-way ANOVA (Table 2.3). Independent of Stage, an increase in total protein was observed in runoff-exposed fish relative to controls. In both control and runoff exposures, there was a consistent increase in K^+ and pCO_2 levels.

Among the most severely affected coho sampled (loss of equilibrium, Stage 4), there was a significant effect of the runoff treatment on blood Na^+ ($p = 0.014$), hematocrit (< 0.001), glucose (< 0.001), and total protein (0.027). None of the blood metrics sampled from fish at Stage 3 when they were behaviorally symptomatic (i.e., surface swimming) showed a significant difference between treatments. There was also no difference in blood chemistry observed for presymptomatic fish (Stage 0) between treatments.

Multivariate analyses (PCA) revealed unique blood profiles specific to urban runoff exposure and behavioral Stages. A summary of PCA results is listed in Table 2.4. From the PCA on the blood metrics for runoff-exposed fish, two principle components (PC) were extracted. PC1 accounted for 39% of the variance in the dataset and PC2 accounted for an additional 23% of the variance (Table 2.4). A difference between Stages was observed along PC2 ($p < 0.001$) and marginally along PC1 ($p = 0.07$) indicating a change in blood profiles as the syndrome advanced. Important blood metrics for explaining the variation between Stages included increased hematocrit, total protein, iCa^{2+}, and pCO_2, and decreased pH and pO_2, indicated by high variable loadings associated with PC1 (> 0.25, absolute value). High loadings for PC2 included a decrease in Na^+, lactate, and osmolality and an increase in pH and K^+. Differences between Stages along the principal components can be visualized in Figure 2.5. Whereas the PCA for runoff-exposed fish showed a progression in blood profiles between Stages, the PCA for control fish did not identify a difference between Stages (Table 2.4).

The PCAs for Stage 0 and Stage 3 did not identify specific blood profiles associated with runoff exposures, as no difference in principal component scores was observed between treatments (Table 2.4). The PCA for Stage 4, however, did identify a specific blood profile corresponding with runoff exposure (Table 2.4; Figure 2.6). For Stage 4, only one principal component was extracted, PC1, explaining 53% of the variance. A significant difference between treatments was identified along PC1 for principal component scores ($p = 0.002$), associated with increased glucose, hematocrit, and total protein, and decreased Na^+ and osmolality. All of the high variable loadings from the PCA of Stage 4 fish were the same blood metrics identified from the two-way ANOVA.

Red blood cell viability was greater than 99% for both control (mean ± SD: 99.5% ± 0.004) and runoff-exposed fish (mean ± SD: 99.2% ± 0.006). No significant difference existed between treatments in fish sampled at Stage 4 for total red blood cell counts ($p = 0.392$). However a significant difference between treatments was observed for the mean corpuscular volume of red blood cells ($p = 0.001$; Figure 2.7).

There was no statistical evidence to suggest that Na^+,K^+ or V-Type H^+-ATPase activity were significantly impaired by urban runoff exposure in kidney or gill tissues (Figure 2.8). However, trends were apparent for increased Na^+,K^+-ATPase activity in gills of Stage 0 (presymptomatic) runoff-exposed fish ($p = 0.124$), and increased V-type H^+-ATPase activity in gills at Stage 4 (loss of equilibrium; $p = 0.055$) and in the tail kidney at Stage 0 ($p = 0.054$).

Conventional water chemistry parameters and metals measured in runoff and control waters collected throughout the study are summarized in Table A.2 and A.3.

2.3 DISCUSSION

Coastal areas of the contiguous United States, including lowland stream and river networks in the Pacific Northwest, are increasingly under pressure from human population growth and associated increases in development, impervious area, stormwater runoff, and degraded freshwater habitat quality. In Puget Sound, adult coho salmon have proven to be an important sentinel species for both toxic stormwater impacts (Scholz et al. 2011; Spromberg et al. 2016; McIntyre et al. 2018) and the effectiveness of clean water mitigation strategies in the form of green stormwater infrastructure (Spromberg et al. 2016). Among Pacific salmonids, adult coho are possibly the most sensitive to toxic runoff, as evidenced by extensive documentation of the urban mortality syndrome both in the field (Scholz et al. 2011; Feist et al. 2017) and under controlled runoff exposure conditions (Spromberg et al. 2016; McIntyre et al. 2018). However, the corresponding impacts of untreated runoff on other coho life stages, and in particular freshwater-phase juveniles, are poorly understood.

This study provides the first detailed analysis of the urban mortality syndrome in juvenile coho, including the temporal progression of behavioral and physiological symptoms in response to experimental exposures to stormwater runoff collected from a high traffic volume arterial in Seattle. As with adults, runoff from this location was almost universally lethal to juvenile coho, and the sequence of behavioral symptoms (e.g., surfacing and gaping > spiral swimming > loss

of equilibrium) was remarkably similar to behaviors widely observed among distressed coho spawners in Puget Sound urban streams (Scholz et al. 2011). The dysregulation of blood chemistry was also similar for the two life history stages, in both cases culminating in severe ion and osmoregulatory impairment as the syndrome progressed. In the future, juvenile coho can provide an experimental platform for investigating the cause of the mortality syndrome, which currently poses a considerable conservation threat to wild coho population segments in Washington and Oregon. To this end, initial mechanistic insights from the current study are discussed below.

2.3.1 *Behavioral indicators of coho in distress*

When surveying freshwater habitats in developed and developing watersheds, there are essentially two methods for positively confirming the coho urban stream syndrome. The first is a forensic examination of carcasses. In the absence of predation, females with most or all of their eggs retained (i.e., unspawned) are definitively pre-spawn mortalities (Scholz et al. 2011). The second method is more elusive, as it requires video documentation of live but symptomatic fish (both males and females). Obtaining this direct evidence is more difficult because encounter rates with live fish in urban streams can be relatively low. Also, affected coho usually die on a timescale of a few hours, and thus field surveys often miss the window of overt symptomology. For these reasons, and the logistical difficulties of working experimentally with wild adult spawners in correspondingly large volumes of stormwater (Spromberg et al. 2016; McIntyre et al. 2018), the progression of behavioral symptoms associated with the mortality syndrome has not been carefully studied.

Until recently, it was also unclear whether the syndrome affected other life stages of coho salmon. Although symptomatic juveniles have been occasionally observed during fall spawner surveys, these encounters have been exceptionally rare (N.L. Scholz, n = 2 unpublished videos). Recently, however, it was shown that direct exposures to highway runoff are also lethal to juvenile coho (McIntyre et al. 2015), setting the stage for the behavioral experiments in the current study.

The sequence of behaviors leading up to juvenile mortality was remarkably consistent, both within and between storms. This allowed for a categorization of severity, or designation of Stages, that could then be used as reference points for evaluating physiological parameters in

blood and tissues. The behavioral reference points also provided a basis for answering, at least provisionally, two key questions.

The first is whether stormwater toxicity to coho is irreversible. The transfer of intermediately distressed fish (i.e., fish swimming at the surface) from stormwater to clean water had one of two likely outcomes: 1) a reversal of the symptomology sequence and the prevention of acute mortality, or 2) a continuation along the sequence, culminating in death. Our results suggest the mortality syndrome is a "one way street" – i.e., coho exhibiting any of the characteristic symptoms in spawning habitats are likely to die. This is significant from a management perspective because storm events and stormwater discharges to coho spawning and rearing areas are intermittent. Thus, a single storm event with high contaminant concentrations may induce an irreversible effect on coho salmon leading to mortality. The cumulative loading of contaminants over the course of several storms may therefore be less important than a single storm degrading water quality to the extent that resident coho become symptomatic.

The second key question is a differentiation between toxic chemical contaminants in stormwater and the substantive differences in salt content between highway runoff and clean reference water. The lack of a behavioral response to synthetic stormwater containing salts at the lower and intermediate range of measured levels in collected runoff (Spromberg et al. 2016) suggests that one or more contaminants coming from roadways is killing coho . From a management perspective, this is helpful in that it narrows the scope of the ongoing forensic search for the causal agents, even if the remaining list of identified and unidentified chemicals in urban stormwater (e.g., Du et al. 2017) is somewhat daunting.

2.3.2 *Physiological correlates of behavioral stress*

The observed behaviors in stormwater-exposed coho also shed some light on potential physiological mechanisms of stress. Specifically, increased surface swimming and gaping suggests that symptomatic fish are starved of oxygen. In runoff-exposed adults, both an ion regulatory and oxygen delivery disturbance was observed (McIntyre et al. 2018).

At the gills, an "osmorespiratory compromise" exists between gas transport and ion balance (Randall et al. 1972; Nilsson 1986). A highly permeable gill with a large surface area is beneficial for maximum gas transfer, but compromises the ability to maintain osmotic homeostasis. Therefore, if oxygen demand is high, osmoregulatory ability is often compromised.

At a loss of equilibrium, runoff-exposed juveniles had evidence of a severe disruption of osmotic balance, but the key indicators of hypoxia (i.e., increase in lactate and pO_2; decrease in pH and pCO_2) were not affected. Runoff-exposed juveniles exhibited a decrease in Na^+ concentration and osmolality, and an increase in red blood cell volume and total protein in the blood. Together, the blood metrics support a reduce blood osmolality and shift in osmotic gradients that could have been induced by an increase in gill permeability. It is therefore possible that respiration in runoff-exposed coho may have been initially maintained by an increase in gill permeability to enhance oxygen delivery, at the consequence of reducing osmoregulatory ability.

The comparable effect of urban runoff on juvenile and adult coho salmon presents the opportunity of using juveniles as a tractable experimental platform for studying the spawner mortality effect observed in the field. The challenge of working with adult spawners, in terms of the availability of fish and the logistics of working with large volumes of stormwater, has not only slowed the progress of research, but also limits the potential questions that may be addressed. Juvenile exposures, however, are not limited to the same degree as adult spawners. Juveniles are convenient to handle, require only a small volume of exposure water compared to adult spawners, and are available year-round in high abundance. Therefore, large juvenile exposures may be conducted year-round to target more precise research questions.

Using juvenile as surrogates for adults, there are several lines of research that may help identify potential mechanisms initiating the urban mortality syndrome. For example, confirming the source of ion loss is necessary to fully interpret the ionic disruption observed. Both branchial and renal ion losses should be monitored throughout the time course of the exposure to identify the time course and source of ion loss. Additionally, measuring hematological indicators, such as blood pressure and total blood volume, would also inform sources of the observed osmotic distress.

From a management perspective, the similar effect of urban runoff exposures on juvenile coho also raises the question of susceptibility of juvenile coho salmon in the field. Juveniles spend a considerably longer time in urban creeks than other Pacific salmonids where they may be exposed to urban stormwater runoff. Compared to adult spawners, juvenile mortality is more difficult to monitor, as juvenile carcasses are smaller, would likely be eaten by predators, and mortality could be occurring year-round. However, lower abundance of juvenile coho has been observed in urban watersheds compared to non-urban ones (Scott et al. 1986; May et al. 1997)

that could potentially be related to the lethal effects of urban runoff observed in this study. If juveniles are similarly susceptible to urban runoff in the field, long-term coho conservation in urbanizing watersheds may be even more challenging than previously predicted (Spromberg and Scholz 2011).

Ultimately, this study provides behavioral and physiological evidence of the urban mortality syndrome in juvenile coho salmon. The toxic constituents responsible for the mortality are unidentified, but have been killing adult coho salmon throughout the region for decades (Scholz et al. 2011; Feist et al. 2017). While the relative susceptibility of juveniles in the field is unknown, the observed lethal effect observed in experimental exposures warrants further investigation of juvenile mortality. Juveniles may also provide the necessary experimental platform to identify the initiating mechanisms of urban runoff toxicity in adult coho salmon. As population growth continues across the Puget Sound region, the impact of urban runoff will increasingly threaten viable coho salmon populations.

In the Pacific Northwest and elsewhere throughout North America, stormwater runoff is the primary driver for non-point source pollution in urban watersheds. Degraded water quality not only changes the quality of the natural ecosystem, but also threatens the sustainability of valued resources including salmonids, shellfish, and clean water. Urban stormwater runoff therefore presents a global challenge throughout metropolitan areas that needs to be quickly addressed to mediate the expansion of the urban stream syndrome.

2.4 TABLES

Table 2.1. Behavioral sequence for the urban runoff-elicited mortality syndrome in juvenile coho salmon.

	Behavior Characteristics
Stage 0	Presymptomatic (no surface activity). Fish categorized by Stage 0 were sampled between 30-60 min after the start of the exposure.
Stage 1	Discrete surfacing events shorter than 0.25 s. Fish swimming within 1 cm of the surface of the exposure tank were scored. Events that only included the tail were not counted.
Stage 2	Short episodes of surface swimming ranging from 0.25-2 s.
Stage 3	Sustained continuous surface swimming. All durations were greater than 2 s and included regardless of swimming pattern (e.g., linear and circular swimming).
Stage 4	Loss of equilibrium including side swimming, inability to stay parallel to the surface, and spiraling.
Stage 5	Loss of buoyancy, as indicated by settlement to the bottom of the tank.
Stage 6	Moribund (near death). Fish in a moribund state exhibited changes in ventilation rates (i.e., gilling), along with spasms and gaping. Moribund coho were also unresponsive to touch.

Table 2.2. Morphometric and blood variables for juvenile coho sampled at specific behavioral stages during runoff exposure along with time-matched controls. Asterisk (*) denotes significant difference from controls at the specific stage. Abbreviations include: CF – condition factor, iCa^{2+} – ionized calcium, HCT – hematocrit, TP – total protein, and Osmo – osmolality.

		Stage 0: Presymptomatic						**Stage 3: Surface swimming**						**Stage 4: Loss of equilibrium**					
		Control			Runoff			Control			Runoff			Control			Runoff		
	Units	n	mean	sd	n	mean	sd	n	mean	sd	n	mean	sd	n	mean	sd	n	mean	sd
Length	mm	15	256.4	27.9	15	251.9	50.8	15	259.5	28.3	15	259.5	65.6	15	256.8	33.4	15	261.2	45.1
Weight	g	15	204.5	67.0	15	203.8	105.6	15	204.5	67.0	15	203.4	105.6	15	219.0	78.5	15	224.2	119.1
CF	kg/cm^3	15	1.17	0.12	15	1.15	0.13	15	1.14	0.13	15	1.07	0.26	15	1.32	0.65	15	1.16	0.24
pH		14	7.31	0.08	14	7.31	0.11	11	7.33	0.11	15	7.32	0.09	14	7.37	0.09	15	7.27	0.12
pCO_2	mmHg	14	10.01	1.10	14	9.27	1.36	11	10.54	1.40	15	11.22	1.98	13	10.64	2.80	13	11.54	3.46
pO_2	mmHg	14	4.21	2.14	14	4.47	1.65	11	3.75	0.90	15	3.38	1.77	13	5.25	2.65	15	4.46	2.08
Na^+	mmol/L	14	139.7	4.75	14	143.5	3.55	11	139.0	5.04	15	137.6	6.16	13	138.2	3.56	15	**131.9***	5.82
K^+	mmol/L	14	4.86	0.79	14	4.26	1.15	11	5.56	1.46	15	5.18	1.30	14	5.24	1.73	15	5.44	1.42
iCa^{2+}	mmol/L	14	1.45	0.08	14	1.48	0.09	10	1.44	0.09	15	1.49	0.13	13	1.43	0.12	14	1.51	0.21
Glucose	mg/dL	13	102.2	14.6	14	103.3	17.6	11	111.7	21.8	14	103.0	26.5	13	102.0	13.6	14	**144.5***	30.8
HCT	%PCV	14	33.8	4.54	13	34.3	8.86	11	31.2	4.96	14	38.9	7.34	14	30.0	7.93	15	**46.9***	12.76
Lactate	mmol/L	13	11.63	5.06	13	10.44	4.26	11	11.68	6.50	12	7.31	5.87	10	3.91	2.26	12	5.80	4.33
TP	mmol/L	13	5.74	0.78	13	5.38	1.47	11	5.18	0.91	12	6.35	0.91	10	5.16	0.76	12	**6.78***	1.73
Osmo	mOsm/kg	13	304	15.45	13	310	17.37	12	309	11.21	12	294	17.39	10	298	7.92	12	285	17.74

Table 2.3. Two-way ANOVAs found significant differences between treatments and/or Stages on blood metrics sampled from juvenile coho salmon. Abbreviations include: iCa^{2+} – ionized calcium, HCT – hematocrit, TP – total protein, Osmo – osmolality.

	Two-Way ANOVA					
	*Treatment		*Stage		Treatment*Stage	
	F	p-value	F	p-value	F	p-value
pH	2.839	0.096	0.284	0.753	1.780	0.175
pCO_2	0.048	0.828	4.009	**0.022***	1.503	0.229
pO_2	0.126	0.724	0.016	0.901	0.374	0.542
Na^+	1.810	0.182	12.923	**<0.001***	7.317	**0.001***
K^+	0.548	0.462	3.292	**0.043***	0.691	0.504
iCa^{2+}	3.558	0.063	0.105	0.900	1.587	0.211
Glucose	3.874	0.050	6.163	**0.003***	8.957	**<0.001***
HCT	17.198	**<0.001***	0.890	0.414	6.371	**0.003***
Lactate	1.804	0.184	18.733	**<0.001***	2.705	0.074
TP	7.148	**0.009***	0.850	0.432	4.636	0.131
Osmo	5.708	**0.025***	8.308	**<0.001***	4.188	**0.019***

Table 2.4. Principal component analysis (PCA) revealed specific blood profiles associated with urban runoff exposures. The significance of each eigenvalue was obtained from Monte Carlo randomization tests. PC significance indicates the significance of principal component scores between treatments or Stages determined by linear regression. Bold indicates high variable loadings for each principal component (> 2.5 absolute value) indicating a high correlation of the blood metric to the principal component. Abbreviations include: iCa^{2+} – ionized calcium, HCT – hematocrit, TP – total protein, Osmo – osmolality.

	Runoff		Control		Stage 0		Stage 3		Stage 4	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Eigenvalue	2.091	1.609	1.826	1.494	1.816	1.599	1.896	1.592	2.411	1.181
% Variance	39.74	23.55	30.32	20.29	29.98	23.25	32.67	23.03	52.86	12.68
P-values: Monte Carlo	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1.000
PC significance	0.069	**<0.001***	0.215	0.515	0.399	0.381	0.341	0.133	**0.002***	0.425
pH	**0.437**	**-0.274**	**0.451**	-0.105	**0.471**		-0.172	**0.452**	-0.166	**0.358**
pCO_2	**-0.440**		**-0.326**	-0.209		**-0.438**	0.144	**-0.444**		**-0.498**
pO_2	**0.258**	-0.133	0.222	-0.159	0.108	0.181	**-0.373**		0.130	**0.485**
Na^+	0.146	**0.480**		**0.612**	**-0.452**	0.206	**0.493**		**-0.428**	
K^+	-0.126	**-0.359**	-0.241	**-0.403**	0.161	**-0.411**	**-0.280**	-0.231		**-0.279**
iCa^{2+}	**-0.402**		-0.119	**0.420**	**-0.345**		0.310	-0.226		**-0.406**
Glucose	-0.197		**-0.394**		**-0.294**	-0.117		-0.207	**0.509**	0.154
HCT	**-0.36**	-0.152	**-0.35**	**-0.314**		**-0.448**	**-0.291**	**0.464**	**0.333**	-0.180
Lactate	-0.181	**0.472**	**-0.396**		**-0.404**		**0.266**	**-0.235**	0.244	
TP	**-0.386**	-0.169	**-0.286**			**-0.569**	-0.191	**-0.416**	**0.299**	-0.210
Osmo		**0.515**	-0.219	**0.317**	**-0.393**		**0.447**		**-0.503**	-0.201

2.5 FIGURES

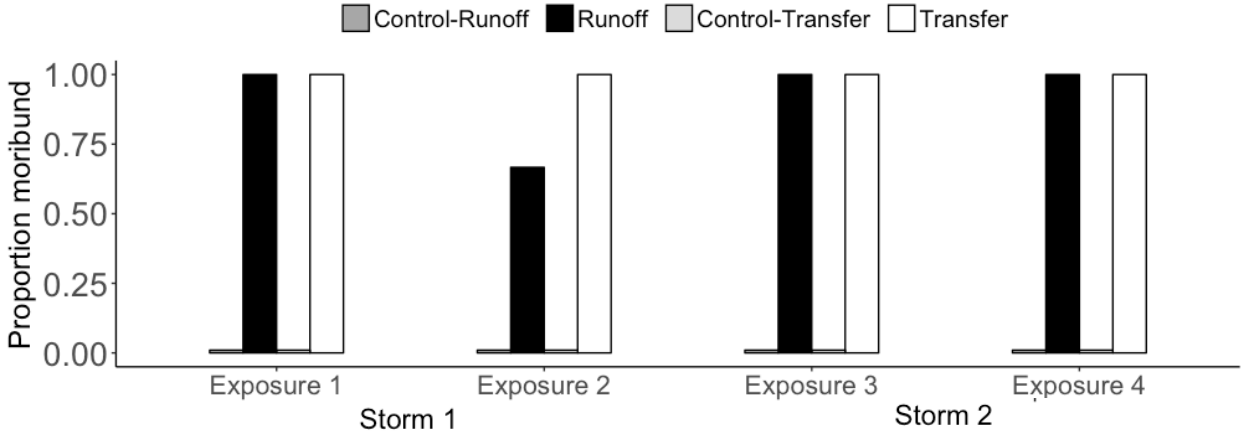

Figure 2.1. Juvenile coho exposed transiently to urban runoff and then transferred to clean water (Transfer) became moribund at the same rate as fish exposed to runoff during the entire experiment (Runoff). There were no time-matched control fish that became moribund from the continuous or transient control exposures. All treatments represent a sample size of 3 fish. Exposure 1 and 2 were conducted using Storm 1 (04/10/2017) and Exposure 3 and 4 conducted using Storm 2 (04/23/2017).

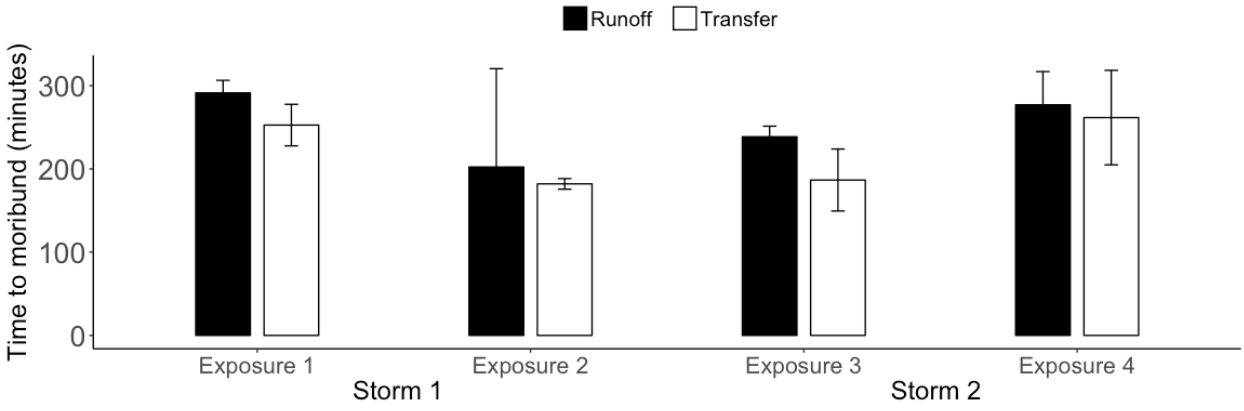

Figure 2.2. Juvenile coho exposed transiently to urban runoff and then transferred to clean water (Transfer) became moribund over the same time course as fish continuously exposed to runoff (Runoff) for each exposure to each storm. Error bars indicate ± one standard error of the mean.

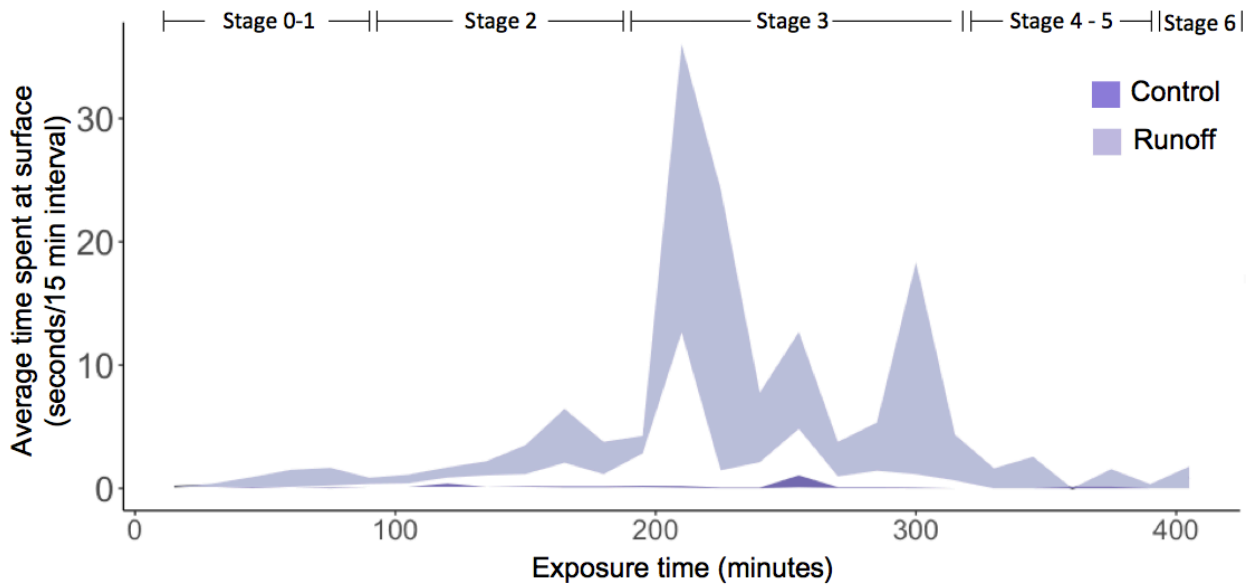

Figure 2.3. Runoff-exposed fish spent significantly more time at the surface relative to coho in clean water. Control fish only exhibited episodic surfacing behavior (< 0.25 s) whereas runoff-exposed fish progressed to periods of continuous surface swimming (> 2 s). Shading captures the standard error of the mean (n = 12-24 per treatment).

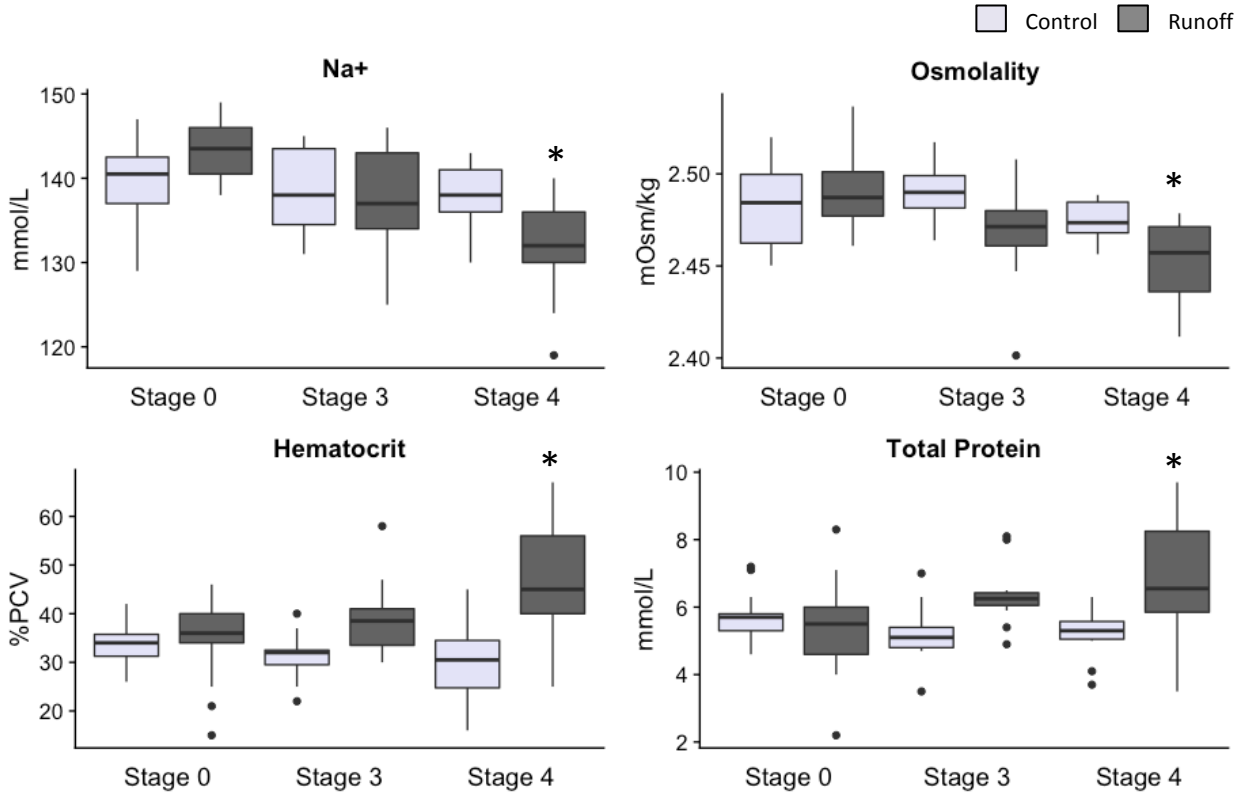

Figure 2.4. Ion and osmoregulatory-related blood variables affected during urban runoff exposure for the three sampling points, representing three stages of the mortality syndrome and corresponding controls for each time point. Abbreviations: mmol – millimole, mOsm – milliosmoles, PCV – packed cell volume. Asterisk (*) indicated a significant difference between treatments at a given Stage. Black dots represent data points greater than 1.5 interquartile ranges (IQR) from the first and third quartiles.

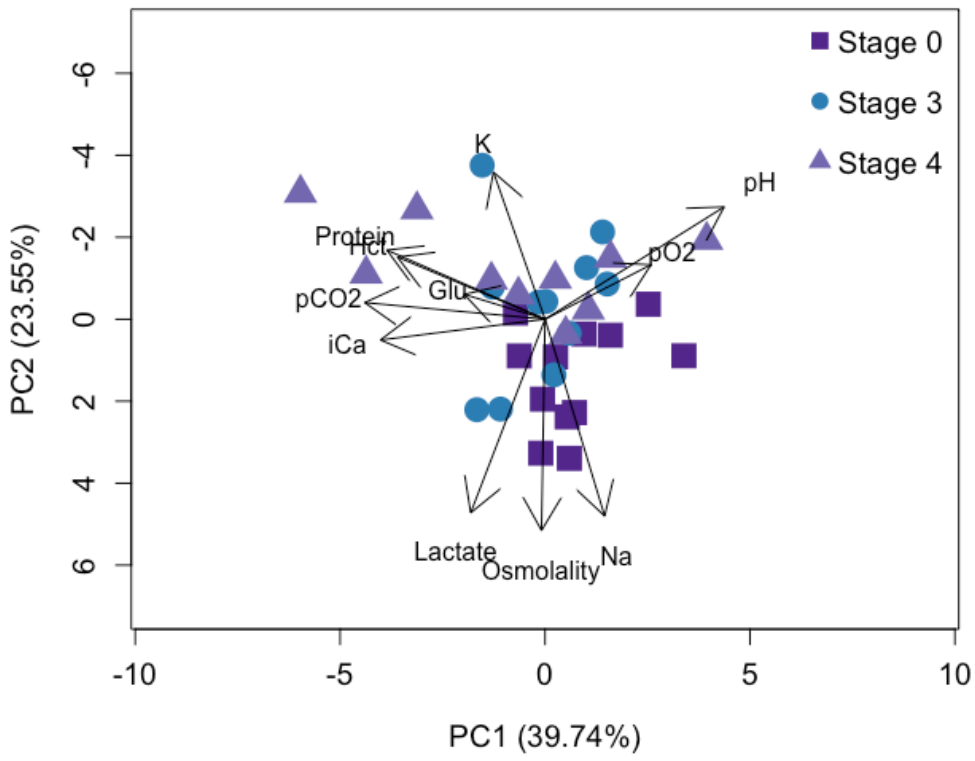

Figure 2.5. Ordination biplot from principal component analysis (PCA) on blood chemistry parameters measured in runoff-exposed juvenile coho, for three stages (Stage 0, 3 & 4) along a gradient of symptom severity. Principal component scores (i.e., location of individuals along principle components) were significantly different along PC2 between behavioral Stages, indicating unique blood profiles associated with Stage.

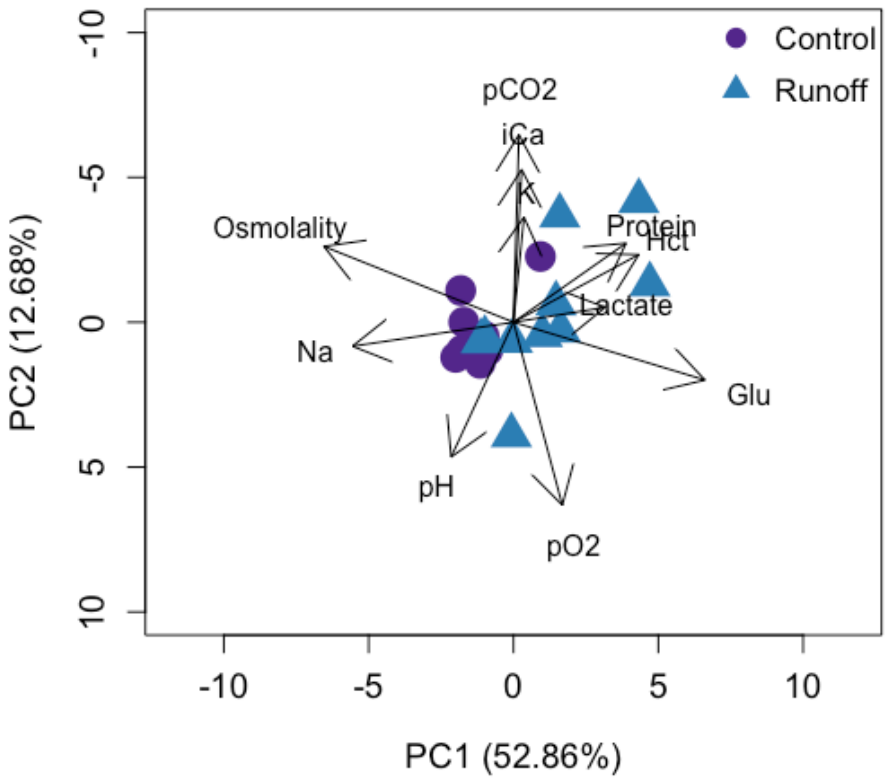

Figure 2.6. Ordination biplot from principal component analysis (PCA) on fish sampled at Stage 4 (loss of equilibrium) indicate a unique blood profile for runoff-exposed juveniles. Principal component scores (i.e., location of individuals along principle components) were significantly different by treatment along PC1 (linear regression; $p = 0.002$) but not PC2. High variable loadings along PC1 (> 0.25 absolute value) include Na^+, osmolality, glucose, hematocrit and total protein.

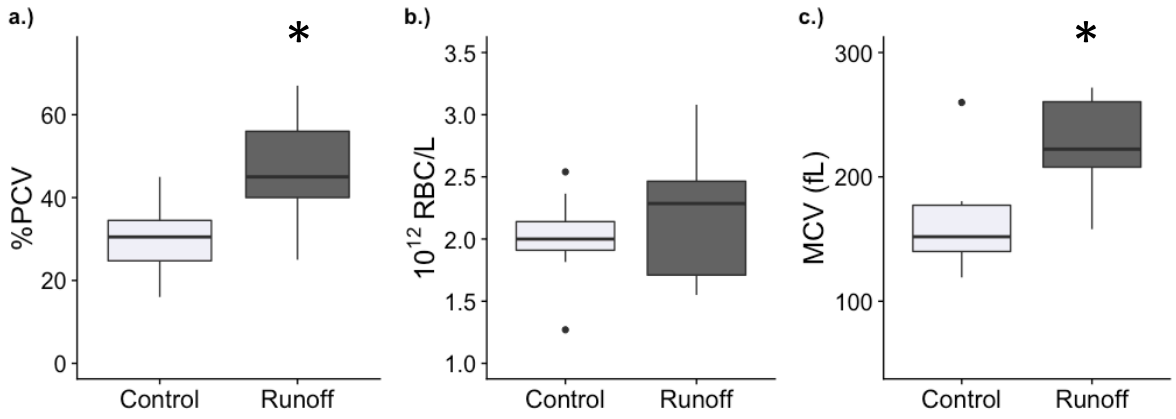

Figure 2.7. Runoff-exposed fish sampled at loss of equilibrium had a significantly higher hematocrit and red blood cell volume compared to time-matched controls: a) Hematocrit as percent packed cell volume (%PCV); b) Total density of red blood cells (RBC); c) Mean corpuscular volume (MCV).

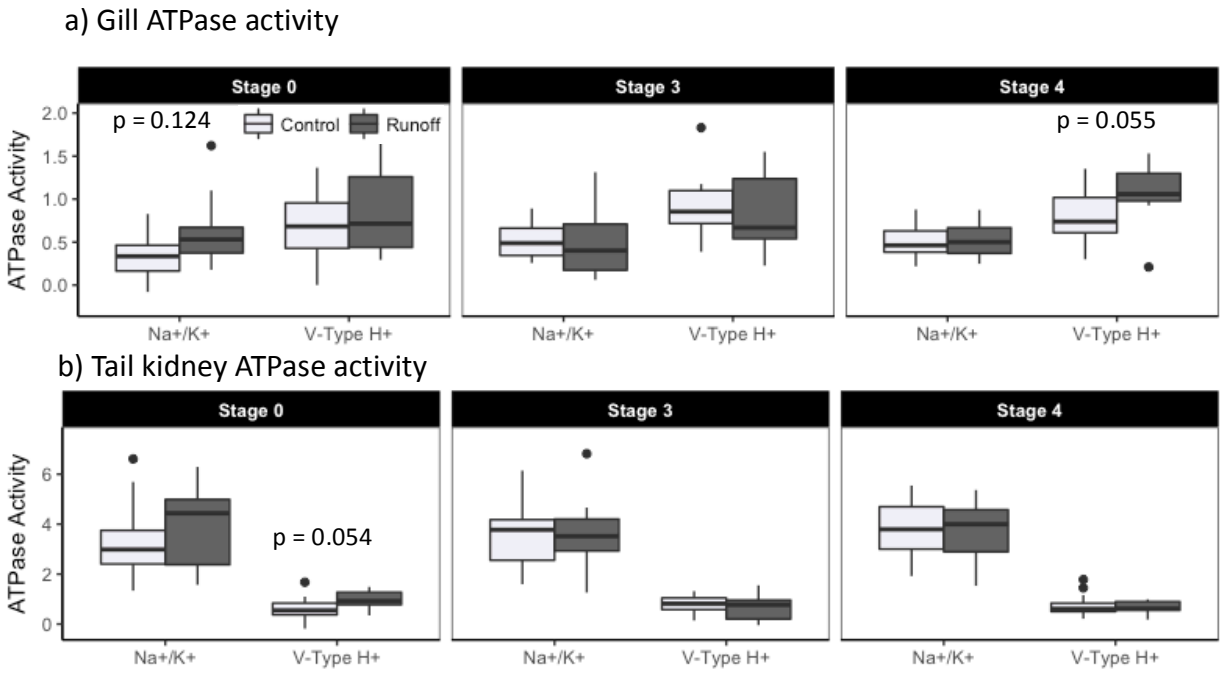

Figure 2.8. Na^+,K^+ and V-Type H^+-ATPase activities were marginally higher in gill and tail kidney tissues of runoff-exposed coho compared to controls. ATPase activity is reported in moles ADP/mg protein/hour: a) Gill ATPase activity. b) Posterior kidney ATPase activity.

BIBLIOGRAPHY

Abbott. 2013a. pO_2 and calculated oxygen saturated/sO_2.

Abbott. 2013b. pCO_2 and calculated values for HCO_3, base excess and anion gap.

Abbott. 2016. pH.

Alberti, M., Booth, D., Hill, K., Coburn, B., Avolio, C., Coe, S., Spirandelli, D. 2007. The impact of urban patterns on aquatic ecosystems: An empirical analysis in Puget lowland sub-basins. Landscape and Urban Planning 80, 345–361.

Bilby, R.E., Mollot, L.A. 2008. Effect of changing land use patterns on the distribution of coho salmon (*Oncorhynchus kisutch*) in the Puget Sound region. Canadian Journal of Fisheries and Aquatic Sciences 65, 2138–2148.

Bolte, J., Vache, K.B. 2010. Envisioning Puget Sound alternative futures. Oregon State University, Corvallis, Oregon, USA.

Booth, D.B., Karr, J.R., Schauman, S., Konrad, C.P., Morley, S.A., Larson, M.G., Burgess S.J. 2004. Reviving urban streams: land use, hydrology, biology, and human behavior. Journal of the American Water Resources Association 40, 1351–1364.

Booth, D.B., Hartlet, D., Jackson, R. 2002. Forest cover, impervious-surface area, and the mitigation of stormwater impacts. Journal of the American Water Resources Association 38, 835–845.

Booth, D.B., Jackson, R. 1997. Urbanization of aquatic systems: Degradation thresholds, stormwater detection, and the limits of mitigation. Journal of American Water Resources Association 33, 1077–1090.

Chowdhury, M.J., Girgis, M., Wood, C.M. 2016. Revisiting the mechanisms of copper toxicity to rainbow trout: Time course, influence of calcium, unidirectional Na+ fluxes, and

branchial Na^+, K^+ ATPase and V-type H^+ ATPase activities. Aquatic Toxicology 177, 51–62.

Du, B., Lofton, J.M., Peter, K.T., Gipe, A.D., James, C.A., McIntyre, J.K., Scholz, N.L., Baker, J.E., Kolodziej, E.P. 2017. Development of suspect and non-target screening methods for detection of organic contaminants in highway runoff and fish tissue with high-resolution time-of-flight mass spectrometry. Environmental Science: Processes and Impacts 19, 1185–1196.

Feist, B.E., Buhle, E.R., Baldwin., D.H., Spromberg, J.A., Damm, S.E., Davis, J.W., Scholz, N.L. 2017. Roads to ruin: conservation threats to a sentinel species across an urban gradient. Ecological Applications 0, 1–15.

Feist, B.E., Buhle, E.R., Arnold, P., Davis, J.W., Scholz, N.L. 2011. Landscape ecotoxicology of coho salmon spawner mortality in urban streams. PLoS ONE 6.

Gallagher, A.J., Frick, L.H., Bushnell, P.G., Brill, R.W., Mandelman, J.W. 2010. Blood gas, oxygen saturation, pH, and lactate values in elasmobranch blood measured with a commercially available portable clinical analyzer and standard laboratory instruments. Journal of Aquatic Animal Health 22, 229-234.

Harrenstien, L., Tornquist, S., Miller-Morgan, T., Fodness, B., Clifford, K. 2005. Evaluation of a point-of-care blood analyzer and determination of reference ranges for blood parameters in rockfish. Journal of American Veterinary Medical Association 226, 255–265.

Katz, S.L., Barnas, K., Hicks, R., Cowen, J., Jenkinson, R. 2007. Freshwater habitat restoration actions in the Pacific Northwest: A decade's investment in habitat improvement. Restoration Ecology 15, 494–505.

Marsalek, J., Rochfort, Q., Brownlee, B., T, M., Servos, M. 1999. An exploratory study of urban runoff toxicity. Water Science and Technology 39, 33–39.

May, C.W., Horner, R.R., Karr, J.R., Mar, B.W., Welch, E.B. 1997. Effects of urbanization on small streams in the Puget Sound ecoregion. Watershed Protection Techniques 2, 483–494.

McIntyre, J.K., Lundin, J.I., Cameron, J.R., Chow, M.I., Davis, J.W., Incardona, J.P., Scholz, N.L. 2018. Interspecies variation in the susceptibility of adult Pacific salmon to toxic urban stormwater runoff. Submitted.

McIntyre, J.K., Edmunds, R.C., Anulacion, B.F., Davis, J.W., Incardona, J.P., Stark, J.D., and Scholz, N.L. 2016. Severe coal tar sealcoat runoff toxicity to fish is prevented by bioretention filtration. Environmental Science and Technology 50, 1570–1578.

McIntyre, J.K., Davis, J.W., Hinman, C., Macneale, K.H., Anulacion, B.F., Scholz, N.L., Stark, J.D. 2015. Soil bioretention protects juvenile salmon and their prey from the toxic impacts of urban stormwater runoff. Chemosphere 132, 213–219.

McIntyre, J.K., Davis, J.W., Incardona, J.P., Stark, J.D., Anulacion, B.F., Scholz, N.L. 2014. Zebrafish and clean water technology: Assessing soil bioretention as a protective treatment for toxic urban runoff. Science of the Total Environment 500–501, 173–180.

Meyer, J.L., Paul, M.J., Taulbee, W.K. 2005. Stream ecosystem function in urbanizing landscapes. Journal of the North American Benthological Society 24, 602–612.

Morley, S.A., Karr, J.R., 2002. Assessing and restoring the health of urban streams in the Puget Sound basin. Conservation Biology 16, 1498–1509.

Moscrip, A.L., Montgomery, D.R. 1997. Urbanization, flood frequency, and salmon abundance in Puget lowland streams. Journal of the American Water Resource Association 33, 1289–1297.

Nilsson, S. 1986. Control of gill blood flow, in: Nilsson, S., Holmgren, S. (Eds.), Fish Physiology: Recent Advances. Croom Helm, London, pp. 87–101.

Oksanen, J., Blanchet, F., Friendly, M., Kindt, R., Legendre, R., McGlinn, D., Minchin, P., O'Hara, B., Simpson, G., Solymos, R., Henry, M., Stevens, H., Szoecs, E., H, W. 2015. vegan: Community 2.4-4., Package.

Paul, M.J., Meyer, J.L. 2001. Streams in the urban landscape. Annual Review of Ecology and Systematics 32, 333–365.

Pess, G.R., Montgomery, D.R., Steel, E.A., Bilby, R.E., Feist, B.E., Greenberg, H.M. 2002. Landscape characteristics, land use, and coho salmon (*Oncorhynchus kisutch*) abundance, Snohomish River, Wash., U.S.A. Canadian Journal of Fisheries and Aquatic Sciences 59, 613–623.

Puget Sound Regional Council. 2009. PART II: Regional Growth Strategy, Vision 2040: The Growth Management, Environmental, Economic, and Transportation Strategy for the Central Puget Sound Region.

R Core Team. 2016. R: A language and environment for statistical computing.

Randall, D.J., Baumgarten, D., Malyusz, M. 1972. The relationship between gas and ion transfer across the gills of fishes. Comparative Biochemistry and Physiology -- Part A: Physiology 41, 629–637.

Regetz, J. 2003. Landscape-level constraints on recruitment of chinook salmon (*Oncorhynchus tshawytscha*) in the Columbia River basin, USA. Aquatic Conservation: Marine and Freshwater Ecosystems 13, 35–49.

Ricker, W.E. 1975. Computation and interpretation of biological statistics of fish populations. Bulletin of the Fisheries Research Board of Canada 401.

Ross, P.S. Kennedy, C.J., Shelley, L.K., Tierney, K.B., Patterson, D.A., Fairchild, W.L., Macdonald, R.W. 2013. The trouble with salmon: Relating pollutant exposure to toxic effect in species with transformational life histories and lengthy migrations. Canadian Journal of Fisheries and Aquatic Sciences 70, 1252–1264.

Scholz, N.L., Myers, M.S., McCarthy, S.G., Labenia, J.S., McIntyre, J.K., Ylitalo, G.M., Rhodes, L.D., Laetz, C.A., Stehr, C.M., French, B.L., McMillan, B., Wilson, D., Reed, L., Lynch, K.D., Damm, S., Davis, J.W., Collier, T.K. 2011. Recurrent die-offs of adult coho salmon returning to spawn in Puget Sound lowland urban streams. PLoS ONE 6.

Schrock, R.M., Beeman, J.W., Rondorf, D.W., Haner, P.V. 1994. A microassay for gill sodium, potassium-activated ATPase in juvenile Pacific salmonids. Transactions of the American Fisheries Society 123, 223–229.

Scott, J.B., Steward, C.R., Stober, Q.J. 1986. Effects of urban development on fish population dynamics in Kelsey Creek, Washington. Transactions of the American Fisheries Society 115, 555–567.

Spromberg, J.A., Baldwin, D.H., Damm, S.E., McIntyre, J.K., Huff, M., Sloan, C.A., Anulacion, B.F., Davis, J.W., Scholz, N.L. 2016. Coho salmon spawner mortality in western US urban watersheds: Bioinfiltration prevents lethal storm water impacts. Journal of Applied Ecology 53, 398–407.

Spromberg, J.A., Scholz, N.L. 2011. Estimating the future decline of wild coho salmon populations resulting from early spawner die-offs in urbanizing watersheds of the Pacific Northwest, USA. Integrated Environmental Assessment and Management 7, 648–656.

Strober, W. 2001. Trypan Blue Exclusion Test of Cell Viability, in: Current Protocols in Immunology. p. 3B:A.3B.1–A.3B.2.

Walsh, C.J., Roy, A.H., Feminella, J.W., Cottingham, P.D., Groffman, P.M., Morgan, R.P. 2005. The urban stream syndrome: current knowledge and the search for a cure. Journal of North American Benthological Society 24, 706–723.

Watkins, J., Ballard, R., Beattie, T., Borone, L., Coleman, J., D'Amato, A., Dickerson, L., Gaffney, P., Hershman, M., Kelly, P., Kosh, C., Muller-Karger, F., Rasmuson, E., Rosenberg, A., Ruckelshaus, W., Sandifer, P., Kitsos, T. 2004. An Ocean Blueprint for the 21st Century. Washington, DC.

Wuertz, D., Setz, T., Chalabi, Y., Maechler, M., Byers, J. 2015. timeDate: Rmetrics - Chronological and Calendar Objects.

APPENDIX

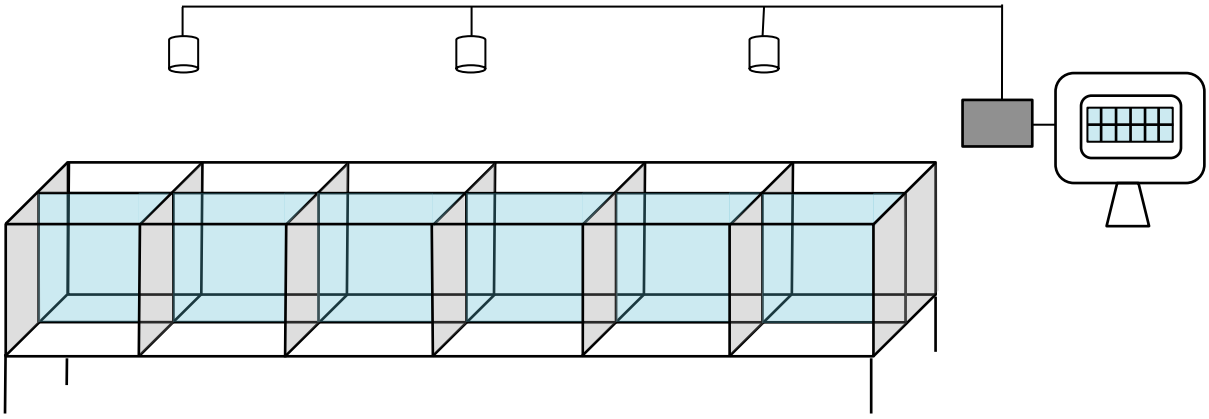

Figure A.1. Acrylic tanks used for behavior and physiology exposures (56.6-L, 61cm x 46cm x 37cm). Three cameras were placed overhead to capture surfacing behavior. Tanks were placed on stands to allow bottom viewing.

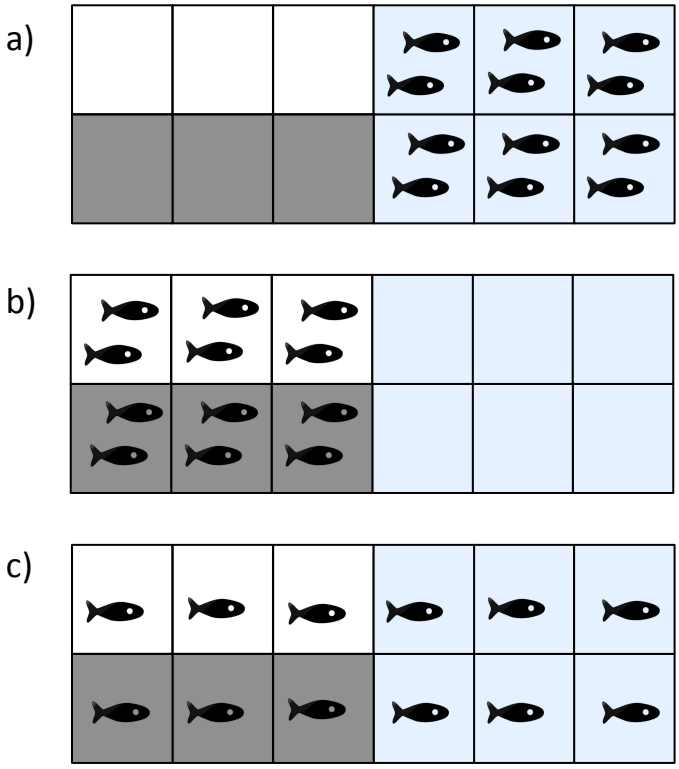

Figure A.2. Exposure sequence for behavioral observations experiments (Section 2.1.5): a) Fish acclimated in clean static water for 2 hr; b) Fish were exposed to clean water or urban runoff; c) At the onset of surfacing behavior in the runoff-exposed fish, one fish from each tank was transferred back to clean water to observe whether recovery was possible. A control fish was transferred with each runoff-exposed fish.

Table A.1. Conventional water quality values used for synthetic stormwater exposures. Control hatchery water is from WSU-Puyallup; Runoff values were calculated from urban runoff collected in 2012 - 2013 (Spromberg et al. 2016).

	pH	**Alkalinity** mg/L $CaCO_3$	**Hardness** mg/L $CaCO_3$
Runoff intermediate	6.8	35.62	77.68
Runoff low	6.4	12.80	20.20
Control hatchery water	7.9	86.00	89.00

Table A.2. Conventional water quality parameters measured in all experimental exposures. Exposures 1-4 indicate behavior studies (Section 2.1.4); Exposure 5 indicates exposure for physiological endpoints (Section 2.1.5). Abbreviations: Temp - temperature; Cond – conductivity, DOC – dissolved organic carbon; TSS – total suspended solids; DO – dissolved oxygen; TAN – total ammonia nitrogen; (-) indicates measures were not measured; ND – parameters were below detection limits.

	Exposure Date	Temp °C	pH	Cond S/m	DOC mg/L	TSS mg/L	TAN mg/L	DO mg/L
Control 1	4/11/17	13.30	7.77	-	0.61	147	ND	10.41
Runoff 1	4/11/17	11.87	7.50	-	15.40	0	8.8	10.70
Control 2	4/12/17	14.30	7.80	-	-	-	-	10.14
Runoff 2	4/12/17	14.13	7.42	-	15.40	171	7.6	10.08
Control 3	4/24/17	14.27	7.93	-	ND	ND	ND	10.23
Runoff 3	4/24/17	13.40	7.69	-	9.55	55	0.37	10.48
Control 4	4/25/17	14.33	7.87	-	-	-	-	10.30
Runoff 4	4/25/17	14.50	7.51	-	9.52	86.5	0.30	10.31
Control 5	6/8/17	18.65	7.78	239	0.79	1.1	0.04	8.29
Runoff 5	6/8/17	17.40	7.60	135	26.30	25.4	0.98	7.60

Table A.3. Metals measured across all experimental exposures. Metals are reported in µg/L. Exposures 1-4 indicate behavior studies (Section 2.1.4); Exposure 5 indicates exposure for physiological endpoints (Section 2.1.5).

		Dissolved metals (ug/L)					
	Exposure Date	Cd	Cu	Pb	Ni	Zn	Ar
Control 1	4/11/17	0.15	1.67	0.20	ND	32	1.30
Runoff 1	4/11/17	ND	28.90	0.53	1.96	6	1.71
Runoff 2	4/12/17	ND	26.70	0.46	1.88	7	1.74
Control 3	4/24/17	ND	3.71	0.22	ND	348	0.99
Runoff 3	4/24/17	0.13	42.80	2.76	1.92	155	3.12
Runoff 4	4/25/17	ND	25.00	0.37	1.43	57	2.87
Control 5	6/8/17	0.25	2.42	0.34	0.50	611	1.03
Runoff 5	6/8/17	0.14	50.30	0.83	2.73	92	4.30

ND = no detection