SALISH SEA

MARINE SURVIVAL PROJECT

Puget Sound Steelhead Marine Survival: 2013-2017 research findings summary

Puget Sound Steelhead Marine Survival Workgroup

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Puget Sound Steelhead Marine Survival Workgroup (all research phases, combined))

Barry Berejikian, NOAA Northwest Fisheries Science Center*
Chris Ellings, Nisqually Indian Tribe*
Ed Connor, Seattle City Light
Erik Neatherlin, Washington Department of Fish and Wildlife *
Jed Moore, Nisqually Tribe
Joseph Anderson, Washington Department of Fish and Wildlife
Ken Warheit, Washington Department of Fish and Wildlife *
Matt Klungle, Washington Department of Fish and Wildlife
Martin Chen, Contractor
Megan Moore, NOAA Northwest Fisheries Science Center
Neala Kendall, Washington Department of Fish and Wildlife*
Paul Hershberger, US Geological Survey, Marrowstone Marine Field Station*
Sandra O’Neill, Washington Department of Fish and Wildlife*
Scott Pearson, Washington Department of Fish and Wildlife
Steve Jeffries, Washington Department of Fish and Wildlife

Contributing Experts: Field Work and Analysis

Adam Hansen, University of Washington
Andrew G. Berger, Puyallup Tribe
Andrea Carey, Washington Department of Fish and Wildlife
Austen Thomas, Smith-Root
Ava Fuller, Muckleshoot Tribe
Bruce Stewart, Northwest Indian Fisheries Commission
Casey Clark, University of Washington
Casey Rice, NOAA Northwest Fisheries Science Center
Catherine Morello, R2 Consulting
Clayton David and crew, Pacific Northwest Salmon Center
Clayton Kinsel, Washington Department of Fish and Wildlife
Correigh Greene, NOAA Northwest Fisheries Science Center
Curtis Nelson, Muckleshoot Tribe
Dave Beauchamp, University of Washington
Eric Jeanes, R2 Consulting
Eric Warner, Muckleshoot Tribe
Eric Ward, NOAA Northwest Fisheries Science Center
Frederick A. Goetz, Army Corps of Engineers
Gary Marston, Washington Department of Fish and Wildlife
Gina Ylitalo, NOAA Northwest Fisheries Science Center

Jesse Nitz, Muckleshoot Tribe
Jason Hall, NOAA Northwest Fisheries Science Center
Josh Chamberlin, NOAA Northwest Fisheries Science Center
Karen Peabody, Washington Department of Fish and Wildlife
Kelly Finley, Tulalip Tribes
Ken Drewes, Muckleshoot Tribe
Kevin Snekvik, Washington State University
Lance Campbell , Washington Department of Fish and Wildlife
Laurie Ann Niewolny , Washington Department of Fish and Wildlife
Madi Gamble, University of Washington
Matt Pouley, Tulalip Tribes
Matthew Klungle, Washington Department of Fish and Wildlife
Mike Crewson, Tulalip Tribes
Mike Mahovlich, Muckleshoot Tribe
Monique M. Lance, Washington Department of Fish and Wildlife
Pete Topping, Washington Department of Fish and Wildlife
Phil Sandstrom, Washington Department of Fish and Wildlife
Rich Henderson and crew, Skagit River System Cooperative
Sayre S. Hodgson, Nisqually Indian Tribe
Sean Hildebrandt, Muckleshoot Tribe
Sean Sol, NOAA Northwest Fisheries Science Center
Steve Damm, US Fish and Wildlife Service
Thomas P. Quinn, University of Washington
Todd Zackey, Tulalip Tribes
Tristan Peter-Contesse, Puget Sound Partnership

<u>Project Management and Facilitation</u>
Iris Kemp, Long Live the Kings
Michael Schmidt, Long Live the Kings**

* Also members of the US Salish Sea Technical Team, the scientific body of the overarching Salish Sea Marine Survival Project.

**For more information, contact mschmidt@lltk.org

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Executive Summary

In 2013, the Washington Department of Fish and Wildlife and the Puget Sound Partnership initiated an effort to determine why juvenile steelhead are dying in Puget Sound.[1] This collaborative effort involves state and federal agencies, Puget Sound Treaty Tribes, nonprofits, and academia. It is coordinated by the nonprofit, Long Live the Kings, and is a component of the Salish Sea Marine Survival Project. The work has been funded by $1.6 million in Washington State appropriations and over $1.0 million in direct match in equipment, services, staff time, and other funding from collaborators.

Through thirteen studies implemented to date, the Puget Sound Steelhead Marine Survival Workgroup determined that the causes of mortality are most likely derived in the lower-river or marine environments and predation and disease are likely the most significant factors affecting survival. In some Puget Sound estuaries, the parasite *Nanophyetus salmincola* is present at high levels and may reduce swimming performance or directly cause mortality. Contaminants in the Nisqually River also negatively impact steelhead health. Compromised fish may be more susceptible to predation, which is likely the immediate cause of most juvenile steelhead mortality within Puget Sound. Harbor seal populations in Puget Sound have nearly tripled since the 1980s, and scat and acoustic telemetry analyses indicate seal predation on juvenile steelhead. Other potential steelhead predators include harbor porpoises and cormorants. In 2016 and 2017, the early marine survival of Nisqually steelhead more than doubled. Initial information suggests that significant changes in the Puget Sound marine environment may have reduced predation risk (e.g. anchovy in high abundance and the presence of transient killer whales). This new information is contributing to our understanding of predation dynamics and factors that may mitigate or exacerbate predation on steelhead populations.

In the next phase of research, the Workgroup will:

1) Continue to assess steelhead early marine survival rates, predation, and factors that may affect the extent of predation including hatchery release magnitude and timing, forage fish abundance, and presence/absence of transient whales.
2) Re-examine the extent to which the *N. salmincola* parasite leads directly or indirectly to mortality.
3) Identify *N. salmincola* hotspots in the Nisqually and Green rivers and recommend actions to reduce their loads.
4) Complete the work to isolate the sources of contaminants in the Nisqually River.

The Workgroup is also currently working with the Puget Sound Steelhead Recovery Team to incorporate their results into the Recovery Plan.

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[1] Steelhead Marine Survival Workgroup. February 2014. Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com

See the “Puget Sound Steelhead Marine Survival: 2017-2019 Research Work Plan”[2] for more information.

The complete list of primary findings are below, embedded in the research question framework of the Workgroup. The studies and their findings are summarized in this report.

Q1. What is the survival history of Puget Sound steelhead and where, when and at what rate is mortality occurring now? How do the abundance and marine survival trends of Puget Sound steelhead populations compare to other regions? How do the abundance trends, marine survival trends, and early marine mortality rates and locations of mortality vary among populations within Puget Sound?

- Puget Sound steelhead population abundance and marine survival have declined and remain lower than other nearby regions.
- Puget Sound steelhead early marine survival rates have been low, with the highest instantaneous mortality rates in South and Central Puget Sound, and the north end of Hood Canal through Admiralty Inlet. Early marine survival increased in 2016, with the greatest reduction in mortality occurring in Central Puget Sound.
- Typically, the farther steelhead must swim through Puget Sound, the greater the mortality (death by distance traveled).

Q2. What is the direct/proximate[3] cause of mortality in Puget Sound?

- A large number of juvenile steelhead are dying quickly in the Puget Sound marine environment, suggesting predation is the source of proximate mortality.
- The list of most likely potential bird and marine mammal predators of outmigrating juvenile steelhead includes harbor seals, harbor porpoises, double-crested cormorants, Caspian terns, and Brandt’s cormorants.
- Harbor seals are a source of proximate mortality in South and Central Puget Sound.

Q3. What is leading to this mortality? What are the root/underlying causes? Are they freshwater and/or marine derived?

- The ultimate source of mortality in Central and South Puget Sound is likely marine derived and not associated with freshwater habitat or hatchery influence. However, causes derived in the lower river or fish condition effects consistent among steelhead populations cannot be ruled out.
- *The parasite, Nanophyetus salmincola,* may kill outmigrating steelhead or make them more vulnerable to predation, contributing to lower early marine survival rates of steelhead

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[2] Puget Sound Steelhead Marine Survival Workgroup. February 2018. Salish Sea Marine Survival Project – Puget Sound Steelhead Marine Survival: 2017-2019 Research Work Plan. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com

[3] The Workgroup defines direct or proximate causes of mortality as those that result in the immediate death of juvenile steelhead.

populations in Central and South Puget Sound. New infections of *N. salmincola* occurring in the lower river are of primary concern.

- PCB's and PBDE's, classes of man-made contaminants, accumulate in some populations of Puget Sound steelhead during freshwater residence, and, coincident with lipid loss, reach levels during smolt outmigration that may affect their health. PBDE's levels in steelhead leaving the Nisqually River are of primary concern.
- Smolts in some populations with particular genetic fingerprints may be predisposed to higher early marine mortality and higher N. salmincola loads. This may be associated with the influence of residency vs anadromy. In some cases, the circadian clock and immune system may also influence parasite loads and survival. However, the power of these findings is limited.
- Juvenile steelhead migrating in April and late May survive at higher rates than steelhead migrating in early-mid May. While not yet investigated, this may be associated with factors like changes in predator-prey dynamics or *N. salmincola* shedding events/disease outbreaks.
- Starvation is not likely. However, we cannot rule out foraging behavior-predation relationships.
- Whole body lipid content was 1.5% or less in the wild Puget Sound steelhead populations that were assessed. Low lipid levels are not inconsistent with the natural decline in whole body lipid content toward depletion during the smolt outmigrant life-stage. However, levels below 1% were observed in some Puget Sound steelhead. This may be cause for concern as 1% is a threshold for the onset of high over-winter mortality in rainbow trout.
- Juvenile steelhead size at outmigration and steelhead outmigrant abundance are not correlated with survival among years. Size at outmigration is also not correlated with survival within years.
- An increase in the abundance of harbor seals correlates with the decline in steelhead survival. Abundance data are lacking for a correlative assessment of the other potential predators; however, qualitative information suggests there may be less of an association with the decline in steelhead survival.
- The presence of alternative or “buffer” prey, when in high abundance, may improve steelhead survival.
- A decline in the abundance of hatchery Chinook, combined with more consolidated release timing of hatchery Chinook subyearlings, may affect predator behavior and make steelhead more vulnerable to predation.
- The presence of transient killer whales may impact harbor seal and harbor porpoise behavior and abundance.
- Increased water clarity and light pollution may exacerbate predation; however, paucity of data limits analyses. Other environmental drivers including Puget Sound sea-surface temperatures and the North Pacific Index may contribute to the factors affecting overall marine survival.

Please visit www.marinesurvivalproject.com for more information.

Introduction

Steelhead trout is the official fish of Washington State, an icon of the Pacific Northwest, and a major contributor to Washington's recreation and fishing economies. Yet the Puget Sound steelhead population, listed as threatened under the Endangered Species Act in 2007, is now less than 10% of its historic size and faces possible extinction. Poor juvenile survival in the Puget Sound marine environment has been identified as a key factor in that decline and a significant barrier to recovery.

Millions of dollars have been spent to recover wild steelhead populations in Puget Sound. Finding a solution to high marine mortality rates of juvenile fish would protect that investment and boost economic activity in communities around the Sound that benefit from viable steelhead fisheries.

In 2013, the Washington Department of Fish and Wildlife and Puget Sound Partnership initiated an effort to determine why steelhead are dying in Puget Sound. Given the level of uncertainty regarding the factors affecting steelhead early marine survival, a multi-disciplinary, ecosystem-based research approach was chosen. To achieve this, the Puget Sound Steelhead Marine Survival Workgroup (Workgroup)[4] was formed, including experts from state and federal agencies, Puget Sound Treaty Tribes, and academic representatives. This Workgroup is coordinated by the nonprofit, Long Live the Kings, and is a component of the Salish Sea Marine Survival Project[5]. The work has been funded by $1.6 million in Washington State appropriations and over $1.0 million in direct match in equipment, services, staff time, and other funding from collaborators.

This report summarizes the findings to date as supporting information for Puget Sound steelhead recovery planning. Extended abstracts of each study are included. *Some studies described in this document are subject to further revisions prior to publication in peer-reviewed journals.* Published studies and technical reports are cited and available on the resources page of www.marinesurvivalproject.com. As manuscripts and reports are completed, they will continue to be made available via the Salish Sea Marine Survival project web site.

Research Framework

To initiate the research program, the Workgroup reviewed, discussed, and categorized the existing evidence and developed their research assumptions based upon the following framework.

Q1. What is the survival history of Puget Sound steelhead and where, when and at what rate is mortality occurring now? How do the abundance and marine survival trends of Puget Sound steelhead populations (hatchery and wild) compare to other Pacific Coast populations, especially other regions of Washington State (e.g., lower Columbia and coast) and the Strait of Georgia? How do the abundance trends, marine survival trends, and early marine mortality rates and locations of mortality vary among populations within Puget Sound?

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[4] Puget Sound Steelhead Marine Survival Workgroup members are listed on the back of the cover of this report.

[5] The Salish Sea Marine Survival Project is a US-Canada research initiative to determine the primary factors affecting juvenile chinook, coho, and steelhead survival in the combined marine waters of Puget Sound and Strait of Georgia. Visit www.marinesurvivalproject.com for more information.

Q2. What is the direct/proximate[6] cause of mortality in Puget Sound?

Q3. What is leading to this mortality? What are the root/underlying causes? Are they freshwater and/or marine derived?

The initial assumptions are summarized in the diagram below (**Figure 1**). Evidence supporting the assumptions is documented in the Research Work Plan: Marine Survival of Puget Sound Steelhead (2014)[7]. In general:

(Q1) Through initial work, the Workgroup found disparate trends and lower smolt-to-adult (marine) survival for Puget Sound steelhead populations compared to those from Washington Coast or the Columbia River. They also found, from acoustic telemetry studies in 2006-2009, that high and rapid juvenile steelhead mortality occurred in Puget Sound. Finally, initial investigations of abundance, smolt-to-adult survival, and early marine mortality data suggested higher mortality for steelhead that travel farther through Puget Sound (those from south Puget Sound or south Hood Canal experience the highest mortality rates).

(Q2) Based on the existing evidence from acoustic telemetry studies showing rapid mortality in Puget Sound, the Workgroup concluded that predation is the most likely proximate source of this mortality. They did not completely rule out other factors that could lead directly to mortality, and concluded those could be assessed peripherally via the studies of ultimate causes of mortality (Q3).

(Q3) While changes in predator abundance could be fundamentally driving steelhead survival in Puget Sound, the Workgroup concluded that a comprehensive assessment of root or ultimate causes was warranted. The Workgroup generally agreed that no one factor is likely working in isolation, and it is the combination of specific factors leading to high mortality rates that must be determined. These ultimate causes/factors were separated into two groups: 1) those that directly affect predator-prey interactions, and 2) those that compromise steelhead condition/health or alter their outmigrant behavior (which could then expose steelhead to higher predation rates or to direct mortality). Factors were further categorized by whether they were freshwater or marine-derived. Based upon existing evidence, the Workgroup then initially ranked causes for poor fish health/altered behavior. Disease was the factor ranked most likely to be compromising steelhead health or altering their outmigrant behavior. Toxic contaminants and a genetic basis for predisposition to mortality were ranked 2nd and 3rd.

Since disease is a broad category, the Workgroup convened fish health experts from the Puget Sound region to prioritize the pathogens and parasites of greatest concern. *Nanophyetus salmincola* was deemed the strongest candidate because of its high prevalence and intensity among other salmonids in the watersheds with the lowest steelhead smolt-to-adult survival rates and highest early marine

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[6] The Workgroup defines direct or proximate causes of mortality as those that result in the immediate death of juvenile steelhead.

[7] Steelhead Marine Survival Workgroup. February 2014. Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com

mortality rates (south Puget Sound and south Hood Canal). *N. salmincola* infections could result in rapid mortality shortly after seawater entry. In particular, the literature shows new infections of *N. salmincola* decrease swimming performance, which could lead to increased predation rates.

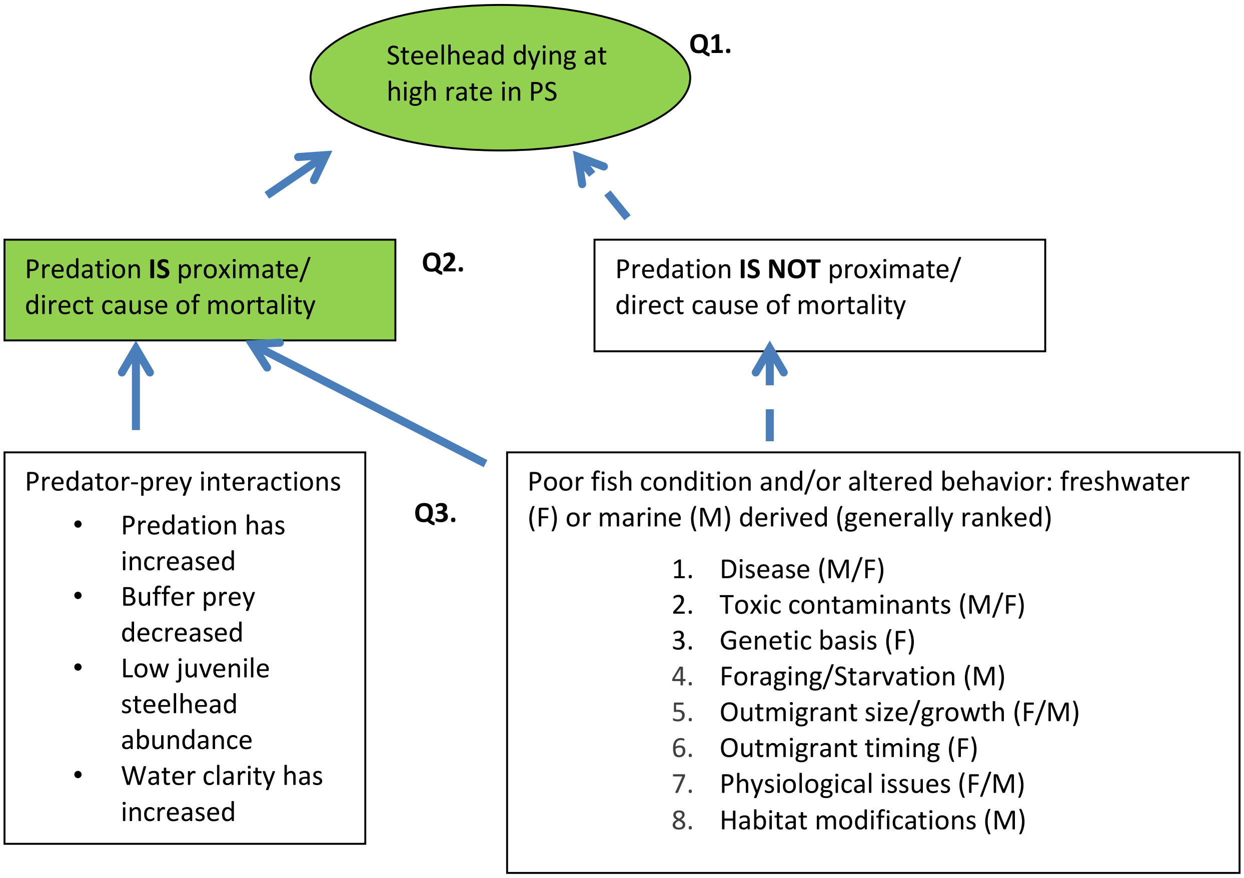

Figure 1. Puget Sound steelhead marine survival research framework and initial evaluation (circa 2013): The green color indicates where the group generally agreed with the evidence. The factors that may be affecting fish condition or behavior are also ranked based upon existing evidence (from Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead (2014)).

Research

Thirteen studies were implemented from 2013-2017 to improve the Workgroup's answers to the three question framework. The studies are categorized in response to the three questions; however, several studies addressed more than one question, as illustrated in the findings section below. Extended abstracts of the specific studies and their findings are provided in the appendices section of this report.

(Q1) The Workgroup concluded additional work should be done to assess the spatial patterns and temporal trends of steelhead mortality using existing abundance, smolt-to-adult (marine) survival, and telemetry/early marine mortality data to: a) establish datasets for assessing correlations with steelhead fish characteristics and environmental characteristics; and b) help further isolate where, when, and at what rate mortality is occurring. Two studies were performed:

- Study 1 (P1)[8]: Multi-population analysis of Puget Sound steelhead survival and migration behavior
- Study 2 (P1): Declining patterns of Pacific Northwest steelhead trout (*Oncorhynchus mykiss*) adult abundance and smolt survival in the ocean

(Q2) A draft list of likely predators was created during this initial round of research, and the Workgroup concluded the list should be formalized through further study of existing data. As harbor seals were considered a strong candidate predator early in the research development process (but not the only predator), the Workgroup concluded that an assessment of harbor seal-steelhead interactions was warranted. Four studies were performed:

- Study 3 (P1): Identifying potential juvenile steelhead predators in the marine waters of the Salish Sea
- Study 4 (P1): Predator-prey interactions between harbor seals and migrating steelhead smolts revealed by acoustic telemetry
- Study 5 (P2:) Interactions between harbor seals and steelhead in Puget Sound, and phase 2 of assessing tag noise effects on survival
- Study 6 (P2): Quantifying juvenile steelhead in harbor seal diet using scat DNA and hard parts analyses in South Puget Sound

(Q3) The Workgroup concluded that the suite of potential factors causing weak steelhead survival could be reduced via a high-level study that helps determine whether the underlying causes of mortality are freshwater or marine derived. They also concluded that existing data could be used to perform a correlative analysis comparing smolt-to-adult survival patterns and trends to steelhead fish characteristics and environmental characteristics. To test the highest ranked factors that may affect fish condition, a assessment of fish health was performed, focusing primarily on *N. salmincola a*nd toxic contaminants and building from the rivers (freshwater) through to the offshore (marine). This was followed by focused work to get a better handle on the ecology of *N. salmonicola* in rivers with high rates of infection. Finally, the Workgroup determined that a genome-wide association study (GWAS) could be performed to determine whether there are genomic differences between outmigrating steelhead smolts that survived to the open ocean versus those smolts that died somewhere within Puget Sound, and the degree to which certain smolts were infected with the *N. salmincola* parasite. The GWAS studies would utilize DNA samples collected from acoustically-tagged smolts in 2006-2009 and 2014 and 2015. Ultimately, 7 studies were performed.

- Study 7 (P1-2): Fish characteristics and environmental variables related to marine survival of Western Washington State steelhead trout (*Oncorhynchus mykiss*)

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[8] (P1) = 2013-2015 Research Phase. (P2) = 2015-2017 Research Phase.

- Study 8 (P1): Population, habitat, and marine location effects on early marine survival and behavior of Puget Sound steelhead smolts
- Study 9 (P1): Steelhead smolt releases from Skagit River used to estimate detection efficiency of Strait of Juan de Fuca acoustic telemetry line
- Study 10 (P1): *Nanophyetus salmincola* infection and toxic contaminant exposure in outmigrating Steelhead Trout from Puget Sound, Washington: implications for early marine survival
- Study 11 (P2): Effects of *Nanophyetus salmincola* on the Swimming Performance and Survival of Steelhead Smolts AND studies to understand and manage the *Nanophyetus* cercaria
- Study 12 (P1): Genome-wide association study of acoustically tagged steelhead smolts in the Salish Sea: measuring differences between survivors and non-survivors
- Study 13 (P2): Genome-wide association study part 2: using (1) survival in acoustically tagged, and (2) Nanophyetus salmincola infested steelhead smolts in south/central Puget Sound, Washington

Findings

The findings of studies are summarized below and organized in accordance with the research framework. For additional details, please reference the extended abstracts for each study in the appendices. Manuscripts or technical reports for each study are or will soon be available at www.marinesurvivalproject.com/resources. Note also that, for the most part, the evidence that established the Workgroup's research assumptions is not repeated below. See "Research Work Plan: Marine Survival of Puget Sound Steelhead"[9] for this complementary evidence.

Q1. What is the survival history of Puget Sound steelhead and where, when and at what rate is mortality occurring now? How do the abundance and marine survival trends in Puget Sound compare to other regions? How do abundance and marine survival trends, and early marine mortality rates and locations of mortality vary among populations within Puget Sound?

Puget Sound steelhead population abundance and marine survival have declined and remain lower than other nearby regions - Spatially-explicit trends in steelhead abundance and smolt-to-adult survival rates (SARs, a.k.a. marine survival rates) were developed for hatchery and wild populations from Puget Sound, the Washington coast and the Columbia River, dating back to the 1970s (study 2). MARSS (Multivariate Auto-Regressive State-Space) models were used to assess whether population dynamics vary among the regions. The results confirmed that Puget Sound populations have distinct trends compared to populations from other nearby regions. Furthermore, Puget Sound steelhead marine

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[9] Steelhead Marine Survival Workgroup. February 2014. Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com

survival rates have generally been lower and have not rebounded as much as populations from other regions. In very recent years, beyond the period assessed in study 2, steelhead marine survival appears to be increasing, but it remains lower than the coast (Kendall, unpublished data).

Puget Sound steelhead early marine survival rates have been low, with the highest instantaneous mortality rates in South and Central Puget Sound, and the north end of Hood Canal through Admiralty Inlet. Early marine survival increased in 2016, with the greatest reduction in mortality occurring in Central Puget Sound - Data collected from several juvenile steelhead telemetry studies that occurred across eight rivers in Hood Canal and Puget Sound were re-examined (study 1). The results indicate early marine survival rates (from river mouth through the Strait of Juan de Fuca) ranged from 0.8% to 39.3%, and averaged 16.0% for wild smolts and 11.4% for hatchery smolts over the four years of the study (2006-2009). Furthermore, results from study 8 indicate early marine survival rates of 5.9 ± 4.2% to 17.4 ± 7.1% for wild steelhead released from the Nisqually and Green rivers, respectively, in 2014. When early marine survival was low, the 2006-2009 data (and 2014 data from study 8) indicate that steelhead smolts suffered greater instantaneous mortality rates (based on a combination of mortality rates and travel distance through specific migration segments) in the south and central regions of Puget Sound and from the north end of Hood Canal through Admiralty Inlet than in other monitored migration segments. In 2016, early marine survival rates for Nisqually wild steelhead increased substantially, to 38% (study 5). Study 5 also revealed a significant increase in survival in Central Puget Sound, from the Tacoma Narrows to the Central Puget Sound telemetry line and the Central Puget Sound to the Admiralty Inlet line: NAR-CPS (85.6 ± 5.6%) and CPS-ADM (80.6 ± 7.4%) migration segments, versus in 2014 NAR-CPS (33.8 ± 6.9%) and CPS-ADM (55.5 ± 8.5%).

Typically, the farther Puget Sound steelhead must swim through Puget Sound, the greater the mortality (death by distance traveled) - Puget Sound steelhead abundance trends support the hypothesis that steelhead survival is worse for populations that have to travel farther through Puget Sound (those entering South and Central Puget Sound compared to populations entering the more northern Whidbey and Rosario basins) (Study 2). This pattern can also be seen in the acoustic telemetry studies (Study 1 and 8), where Nisqually and Skokomish steelhead--in Puget Sound and Hood Canal, respectively—experience the lowest early marine survival rates. In study 8, the location of the river mouth within Puget Sound had the greatest bearing on survival of steelhead smolts through Puget Sound; smolts with shorter migration distances survived at a higher rate than those with longer distances to migrate. Finally, in study 9, an assessment of the early marine survival rates of Skagit steelhead showed that fish taking the shorter migration route to the Pacific Ocean, through Deception Pass and the Strait of Juan de Fuca (119km), survived at 1.7 times the rate of those that took the longer, southern migration route through Saratoga Passage, around Whidbey Island and then northwest through Admiralty Inlet and the Strait of Juan de Fuca (207km).

In study 9, V7 tag detection efficiency of the Strait of Juan de Fuca telemetry line was also tested, comparing the standard tags used in most of the steelhead studies (V7 tags) to larger, more powerful tags that have 100% detection efficiency (V9 tags). The results showed that 66.7% of the V7 tags were detected by the Strait of Juan de Fuca line. The agreement of this empirical estimate with modeled line efficiency rates of 68.5% (Melnychuk 2009)[10] increased confidence in survival estimates based on V7 tag studies.

Q2. What is the direct/proximate cause of mortality in Puget Sound?

A large number of juvenile steelhead are dying quickly in the Puget Sound marine environment, suggesting predation is the source of proximate mortality – Studies 1, 5, 8 and 9 showed juvenile steelhead travel rapidly through the estuary and marine environments of Puget Sound. In Study 1, average migration times from river mouth through the Strait of Juan de Fuca ranged from only 6.2 days (Green River population) to 18.1 days (Skokomish River population). In study 8, travel times from river mouth through the Strait of Juan de Fuca were – A) Nisqually releases: 257 km in 9.80 ± 1.19 days. B) Green releases: 187 km in 8.80 ± 0.44 days). In study 5, travel times for the Nisqually releases were 10.60 ± 0.71 days. In study 9, the average travel time for Skagit steelhead smolts from Skagit Bay through the Strait of Juan de Fuca was 5 days. Rapid outmigration rates, coupled with high freshwater survival and low Puget Sound marine survival rates, suggest a source of mortality that acts quickly on a large number of smolts in the Puget Sound marine environment. Predation fits this pattern well. Substantial indirect evidence from studies 4, 5, and 8, described in further detail below, supports predation as the proximate source of mortality.

Other sources of proximate, instantaneous mortality could include contaminants, harmful algae blooms, or disease. However, based upon the results of the study 10, it is unlikely that contaminants cause direct mortality. Contaminant levels in outmigrating Puget Sound steelhead are lower than mortality thresholds (study 10). Study 10 found that the prevalence and intensity of *Nanophyetus salmincola* infections are high for juvenile steelhead outmigrating from the Nisqually and Green rivers. A laboratory study in 2014 did not provide any indication that moderate *N. salmincola* loads would result in instantaneous mortality during seawater transition. However, the logistics of the laboratory study resulted in a lag of three weeks between *N. salmincola* exposure and seawater challenge, with the experimental seawater transition occurring after the first 14 days, the most pathogenic stages of infection. The laboratory study was tried formally in 2016 (study 11). Mortality was slightly higher among infected fish (6.7%) than among uninfected cohorts (0%); however, the differences were not significant. The parasite loads produced in the 2016 lab study were ten times lower than the loads of steelhead migrating down the Nisqually River; therefore, this study will be repeated.

Finally, as stated in the Puget Sound steelhead marine survival research work plan for 2014[11], acoustic telemetry and SAR data indicate that mortality is not highly variable on an inter-annual basis and occurs throughout Puget Sound, suggesting that mortality is not caused by factors with high spatial and temporal variability in the environment such as harmful algae blooms. Additional retrospective work was recommended to support or refute this hypothesis. However, data are limited.

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[10] Melnychuk MC (2009) Mortality of migrating Pacific salmon smolts in Southern British Columbia. PhD thesis. University of British Columbia, Vancouver.

[11] Steelhead Marine Survival Workgroup. February 2014. Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com

The list of most likely potential bird and marine mammal predators of outmigrating juvenile steelhead includes harbor seals, harbor porpoises, double-crested cormorants, Caspian terns, and Brandt's cormorants - Potential marine mammal and bird predators of out-migrating juvenile steelhead were identified in study 3 based on predator distribution, abundance, and diet information. Based upon the literature review, harbor seals, double-crested cormorants, Caspian terns, and Brandt's cormorants are the most likely potential predators. These fish-eating species have demonstrated relatively stable or increasing population trends in recent years (over the same period as the decline in Puget Sound steelhead marine survival) and their diet includes juvenile salmon and steelhead.

Double-breasted cormorants may be of lower concern because a large portion of the population migrates to the Columbia River in late April for mating season, before the peak of the juvenile steelhead outmigration period. Although, it is possible that immature birds (one and two- year olds) may linger in the Sound longer than adults since they do not fully populate the Columbia River breeding colonies until mid-June. Anecdotally, the presence of Caspian tern nesting has been variable in Puget Sound in recent years, and the May nesting period coincides with steelhead outmigration.

The abundance of harbor seals has increased substantially in Puget Sound and the greater Salish Sea over the period of steelhead decline. Study 7 illustrates the strong inverse relationship between seal abundance and Puget Sound steelhead marine survival. The relative abundance and distribution of harbor seals during the April-June steelhead outmigration period has not been established; however, it is a priority.

Harbor porpoise sightings have increased dramatically in Puget Sound through the 1990s and 2000s (Study 3). The increase in harbor porpoise sightings was greatest from the late 1990s onward, after the period during which Puget Sound steelhead marine survival declined significantly (study 2). However, the harbor porpoise data over the period of steelhead decline are coarse (study 3). Porpoises find their prey using echolocation allowing them to exploit a resource like juvenile steelhead that tend to move individually or in small groups. However, no salmon or steelhead have been present in diets of Salish Sea harbor porpoise, despite reasonable sample sizes for April and May, the period of juvenile steelhead outmigration (Walker et al. 1998, Nichol et al. 2013, as referenced in study 3).

Recent dive data from sea lions in South Puget Sound during the steelhead outmigration period suggest that sea lions are mainly foraging deep in the water column, at lower depths than where juvenile steelhead outmigrate.[12]

Harbor seals are a source of proximate mortality in South and Central Puget Sound – Study 4, performed in 2014, investigated predator-prey interactions between harbor seals and juvenile steelhead migrating through Puget Sound using acoustic telemetry (246 tagged steelhead and 11 seals with mounted receivers). The study resulted in the first data suggesting harbor seals consume juvenile steelhead in Puget Sound. The study showed that harbor seals and migrating steelhead have substantial spatial and temporal overlap, and study 4 provided indirect evidence of harbor seal predation events via tag detection patterns (repeated detections over 3-4 day period consistent with gut passage time for

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[12] pers. comm. S. Jeffries, Washington Department of Fish and Wildlife, June 2015.

harbor seal, tag movement consistent with seal behavior, and tags detected as stationary (deposited) near harbor seal haulout sites). Detection data did not suggest any tagged smolts were ingested by seals with mounted receivers, and we cannot rule out that tags may have been deposited near haulout sites by other predators. This study was repeated again in 2016 but with increased tag detection capacity (study 5): 199 steelhead were tagged and 16 seals with mounted receivers along with additional fixed hydrophones moored in Puget Sound and mobile tracking post-migration for stationary tags. Steelhead survival through Puget Sound was much higher in 2016, and fewer tags were found near seal haulouts. However, numerous tags that ultimately ended up stationary exhibited movement consistent with seal behavior.

Study 8, which also assessed acoustically-tagged steelhead, provided additional indirect evidence of predation by harbor seals. Not described in the abstract appended to this document, but in the manuscript, some of the acoustic tags were detected moving back and forth with the tides, through the Nisqually estuary and nearby marine environment, and not detected again by the receiver arrays in Puget Sound. This pattern is consistent with harbor seal behavior in estuaries, suggesting the tagged steelhead were consumed by a harbor seal and the harbor seal was detected by the receivers in the estuary and marine environment.

In study 6, steelhead were found in seal scat in South Puget Sound. Steelhead occurred in only one temporal stratum (May 1 – 15) during the 7-8 week steelhead outmigration period, and comprised 1.5% of the harbor seal population diet during that stratum. This is a relatively low value when considering the entire outmigration period. However, steelhead experienced anomalously high early marine survival in 2016. Further, sample sizes were below the amount considered necessary to adequately capture the consumption of a prey type that naturally occurs in very small abundances relative to other seal prey.[13] This work is being repeated in 2017 and 2018, targeting increased sample sizes.

These studies have not resulted in an estimate of the overall predation rate by seals on migrating juvenile steelhead.

The potential for a dinner bell effect (pinging tags attracting harbor seals and biasing results) was also tested in study 4 and again in study 5. There was no evidence for effects of tag noise on survival of steelhead smolts.

Q3 What is leading to this mortality? What are the root/underlying causes? Are they freshwater and/or marine derived?

As stated above, ultimate causes/factors were investigated in two groups: 1) those that directly affect predator-prey interactions, and 2) those factors that compromise steelhead condition/health or alter their outmigrant behavior (which could then expose steelhead to higher predation rates or to direct mortality). Factors were further isolated by whether they were freshwater- or marine-derived.

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[13] A prey population comprising only a small percentage of the seal diet could still be heavily impacted, especially if that population is depressed as steelhead are.

The ultimate source of mortality in Central and South Puget Sound is likely marine-derived and not associated with freshwater habitat or hatchery influence. However, causes derived in the lower river or fish condition effects consistent among steelhead populations, cannot be ruled out – Study 8 took advantage of contrasting conditions in geographically proximate river systems to test for the effects of freshwater rearing conditions and hatchery introgression on survival rates of steelhead migrating from river mouth to the Pacific Ocean (Green River = degraded habitat and current hatchery influence, Nisqually River = high quality habitat and no current hatchery steelhead influence). Steelhead smolts were cross-planted from one river to another and compared to plants into natal rivers to determine whether low early marine survival rates could be due to population-specific effects like freshwater rearing conditions or hatchery introgression, or if direct effects within the marine environment were more likely the cause. Similar survival probabilities occurred among smolts released in the Green and Nisqually rivers, despite clear differences in freshwater habitat and hatchery influence, rendering these factors unlikely to substantially influence early marine survival of these populations. However, because the fish were released at river kilometer 19 in both systems, factors affecting steelhead in the lower river, if immediate and at a high rate (e.g., disease and contaminants), could still explain similarities in mortality of the two reciprocally transplanted populations. Furthermore, although less likely, underlying drivers of fish condition could be the root cause of, or contributing to, the mortality. However, these underlying drivers would have to be consistent among populations to pair with the results of study 8.

Fish condition, the factors affecting condition or altering behavior, and their potential role in juvenile steelhead mortality

The parasite, *Nanophyetus salmincola,* may kill outmigrating steelhead or make them more vulnerable to predation, contributing to lower early marine survival rates of steelhead populations in Central and South Puget Sound. New infections of *N. salmincola* occurring in the lower river are of primary concern. – Study 10 compared the prevalence and intensity of *N. salmincola* and other diseases in five steelhead populations throughout Puget Sound (Skagit, Snohomish, Green, Nisqually) and Hood Canal (Tahuya). The prevalence and parasite loads of *N. salmincola* were significantly higher in outmigrating steelhead smolts from central and south Puget Sound watersheds (Green and Nisqually) than in those from north Puget Sound (Skagit and Snohomish), where infections were rarely detected. *N. salmincola* was also not found in any smolts from the Tahuya watershed. The Green and Nisqually Rivers had high prevalence and parasite loads (above reported thresholds for negative health effects), and a substantial portion of fish from these rivers with *N. salmincola* also exhibited gill (Green 28%, Nisqually 45%) and heart (Green 45%, Nisqually 63%) inflammation not found in the other three rivers. A downstream progression of *N. salmincola* prevalence and intensity in steelhead, and high prevalence and intensity of *N. salmincola* in steelhead captured in the estuaries, suggests that new infections of *N. salmincola* may be occurring as juvenile steelhead move downstream and out into Puget Sound during their migration. Alternatively, these results may indicate that outmigrants with heavy *N. salmincola* loads tend to linger and accumulate in the estuary, rather than outmigrating through Puget Sound with their healthier cohorts. Furthermore, substantial differences in *N. salmincola* prevalence between Green (13.3%) and Nisqually (98-100%) steelhead captured at the in-river trap sites combined with the results of study 4 (similar early marine survival rates of steelhead captured at these trap sites and reciprocally transplanted) further suggest that host survival may be influenced by novel *N. salmincola* exposures that occur in the lower portions of the watersheds. The presence of new infections occurring in the lower river/estuaries of the Green and Nisqually, and heart and gill inflammation found in the steelhead, may be killing the steelhead outright in Puget Sound or, more likely, compromising their ability to swim as they enter and migrate through Puget Sound and increasing their susceptibility to predation.

Finally, histology was performed to investigate the prevalence of other disease conditions in study 8. While other pathogens were found, none other than *N. salmincola* were considered to be consistent with Puget Sound early marine mortality patterns.

It should be noted here that *N. salmincola* does not explain the early marine mortality rates experienced by steelhead in Northern Puget Sound, or those in the Strait of Georgia. Furthermore, based upon the results of study 2, there is a stronger association in patterns of smolt-to-adult survival between North Puget Sound and Central & South Puget Sound populations than between North Puget Sound and coastal or Columbia River populations.

The degree to which *N. salmincola* contributes to steelhead mortality was assessed in study 11. This was done in part by comparing the survival of steelhead infected with the parasite versus steelhead that remained, through Puget Sound and the Strait of Juan de Fuca. This work was performed by infecting the treatment group of fish in the lab, inserting hydroacoustic tags (Vemco V-7) into smolts from both groups, then releasing the fish and recording tag detections at three stationary hydrophone arrays located along the outmigration corridor. While the infected fish did die at a higher rate, the survival comparisons between each group to each of the arrays were not statistically significant. Laboratory tests of transitioning steelhead to saltwater and evaluating their swimming performance both showed similar results: infected steelhead mortality was 6.7% vs 0% for uninfected in the saltwater challenge and there was a slight inverse relationship between swimming performance and parasite load. However, the parasite loads achieved for the treatment groups for all three experiments were ten times lower than witnessed in the wild. These experiments will be repeated in 2018, with more focus put on achieving higher parasite loads.

PCB's and PBDE's, classes of man-made contaminants, accumulate in some populations of Puget Sound steelhead during freshwater residence, and, coincident with lipid loss, reach levels during smolt outmigration that may affect their health. PBDE's levels in steelhead leaving the Nisqually River are of primary concern – Study 10 investigated contaminant loads in three of the Puget Sound steelhead populations screened for parasites in 2014: Skagit, Green, Nisqually. The Snohomish, Hood Canal, and Tahuya populations were not included. Results show that man-made, persistent organic pollutants are generally below concentrations associated with adverse effects. PCB and PBDE[14] levels did exceed potentially harmful levels up to 17-25% and 50%, respectively, of samples from steelhead recovered in the North/Whidbey Basin, Central and South Puget Sound offshore marine habitats. However, PCB concentrations were low within the Skagit, Green and Nisqually rivers and their associated estuaries. The increase in harmful PCB concentrations offshore is primarily due to lower fish lipid content as migration proceeded. In contrast, 33% of the steelhead collected in the in-river trap and the estuary of the Nisqually River had PBDE levels that could increase disease susceptibility or alter thyroid production.

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[14] A type of flame retardant.

Nisqually steelhead were again analyzed in 2015 and the same results were found. However, to be consistent with the results of study 8 that suggest freshwater habitat isn't affecting early marine survival, Nisqually steelhead may need to be impacted by these PBDEs rapidly via exposure below river kilometer 19, the release site for study 8. Regardless, due to the persistent levels seen and their known to impact salmonid health, the source of these contaminants is being pursued so that it can be addressed.

Smolts in some populations with particular genetic fingerprints may be predisposed to higher early marine mortality and higher *N. salmincola* loads. This may be associated with the influence of residency vs anadromy. In some cases, the circadian clock and immune system may also influence parasite loads and survival. However, the power of these findings is currently limited. – Two rounds of genome-wide association studies (GWAS) were performed to test the hypothesis that there is a genomic association with (1) survival of outmigrating steelhead smolts as they transit from through Puget Sound to the Pacific Ocean, or (2) *Nanophyetus salmincola* infestation in steelhead smolts captured in the freshwater, estuary, or offshore areas (Studies 10 and 11). These studies were performed by analyzing DNA samples taken prior to release of acoustic-tagged steelhead in past years. The pilot year (study 10) included tagged fish multiple watersheds, then paired down to the Nisqually, Green and Skokomish rivers after removing sample sets that may confound the results. The results suggested that survival may be influenced by differences in morphological features that may affect swimming performance (axial and fin development) and in the capacity for a fish to respond to pathogens or parasites). The many rivers and therefore lineages and collection years, and the few individuals that were categorized as survived, created small samples sizes and limited statistical power. In study 11, the sample design was improved by limiting analyses to two rivers (Green and Nisqually) and two years (Green + Nisqually = 2014 and Nisqually = 2015) with higher sample sizes. An additional data set characterizing *Nanophyetus salmincola* loads in 2014 was also included. From both the survival and *Nanophyetus* data sets, there is a genomic association with both steelhead smolt survival and *Nanophyetus* infestation, but the association is statistically weak. The strongest association is with Omy05 genotypes, known elsewhere to be related to residency (A allele) vs anadromy (R allele). If the Omy05 genotypes here are associated with migration life histories, it is possible that the Omy05 A allele is maintained in the anadromous steelhead population by resident rainbow trout, and the presence of that A allele may reduce the individual's probability of survival or will result in a higher *Nanophyetus* count, which directly or indirectly may reduce survival. That the Omy05 association is seen in both the Green and Nisqually population provides a basis for consistency with the outcomes of the reciprocal transplant study (study 4). Other components of the genome are more difficult to discern and appear population specific (e.g. loci associated with the circadian clock and a locus associated with the immune system in the Nisqually River). While this work lacked sufficient statistical power, steelhead early marine survival does appear to be associated with a smolt's genome. Further work would be needed to understand the importance of the genome compared to environmental factors and how the genome interacts with environmental factors. In particular, further assessing the relationship between the genome, nanophyetus loads and smolt survival could be promising.

In low early marine survival years, juvenile steelhead migrating in April and late May survive at higher rates than steelhead migrating in early-mid May. While not yet investigated, this may be associated

with factors such as changes in predator-prey dynamics or *N. salmincola* shedding events/disease outbreaks - Based upon the results of study 1, outmigration timing was an important factor driving wild steelhead smolt early marine survival. Steelhead smolts migrating in early April and late May had a higher probability of survival than those released in early and mid-May. Furthermore, study 8 showed that steelhead from the Nisqually population migrating earlier (late April) survived better than those migrating later (though no difference in survival by release date was observed for smolts of Green River origin). This could be associated with several different factors. Predators may not be responding to the steelhead outmigrants until the peak of the steelhead outmigration period or when hatchery coho, steelhead and Chinook are released in large numbers. Alternatively, earlier (or later) outmigrants may avoid *N. salmincola* shedding events in the lower river. In 2016 (study 5), Nisqually steelhead early marine survival was much higher and this within year mortality pattern was not apparent.

Starvation is not likely. However, we cannot rule out foraging behavior-predation relationships –See the description on p. 25 of the Workgroup's initial research work plan regarding steelhead foraging behavior and the unlikelihood of starvation.[15] Telemetry data are generally inconsistent with what we would assume to be steelhead foraging behavior (indicated by vertical or back and forth movement at the telemetry receiver arrays), and the rapid outmigration rate and uniform direction of migration is consistent with steelhead outmigration patterns in other regions with higher steelhead survival, such as the Columbia River estuary. Further, juvenile steelhead migration rates and behavior through Puget Sound, comparing low and high early marine survival years, don't appear to differ (Nisqually 2014 = 9.80 ± 1.19 days and Nisqually 2016 = 10.60 ± 0.71 days). Similar results are found when comparing diet analyses between Puget Sound and the Columbia River estuary. In 2014, in offshore areas of Puget Sound, a higher proportion of steelhead had empty stomachs in central Puget Sound (78% empty, N = 9) and South Sound (50% empty, N = 6) versus Whidbey Basin (29% empty, N = 55) (Kemp, unpublished data 2018). However, the sample sizes were small. Similarly, about 50% of the steelhead had empty stomachs in the Columbia River estuary. Off the coast, they ate more (only about 10% empty), supporting the notion that steelhead are not focused on feeding in estuary environments (Daly et al. 2014).[16] Questions remain regarding what triggers marine phase feeding and when. Without knowing, it's difficult to conclude that steelhead are not interested in foraging in the offshore of Puget Sound. For example, continued rapid migration through Puget Sound could be induced by a lack of food in a particular area and could lead to increased exposure to predation.[17] Therefore, forage-induced predation cannot be ruled out.

Whole body lipid content was 1.5% or less in wild Puget Sound steelhead populations that were assessed. Low lipid levels are not inconsistent with the natural decline in whole body lipid content toward depletion during the smolt outmigrant life-stage. However, levels below 1% were observed in

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[15] Steelhead Marine Survival Workgroup. February 2014. Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com.

[16] Elizabeth A. Daly, Julie A. Scheurer, Richard D. Brodeur, Laurie A. Weitkamp, Brian R. Beckman & Jessica A. Miller (2014) Juvenile Steelhead Distribution, Migration, Feeding, and Growth in the Columbia River Estuary, Plume, and Coastal Waters, Marine and Coastal Fisheries, 6:1, 62-80, DOI: 10.1080/19425120.2013.869284

[17] pers. comm. C. Walters December 2014. December 2014 Salish Sea Marine Survival Project, US-Canada Retreat.

some Puget Sound steelhead, and this may be cause for concern as 1% has been documented as a threshold for the onset of high over-winter mortality in rainbow trout – Whole body lipid content was analyzed in wild steelhead in study 10 as a metric of fish condition. The results indicate that the pooled samples analyzed had levels at or less than 1% for three rivers (Nisqually, Green, Skagit) assessed. Low lipid levels are a natural function of the spring smolt outmigrant life stage. During the smolt stage, energy is heavily used for growth and migration vs. stored as fat, and there is a decline in whole body lipid content toward depletion.[18,19] However, smolt lipid levels lower than 1% were not documented in the papers reviewed.[20,21,22] Lipid levels below 1% have been associated with the onset of high over-winter mortality in rainbow trout.[23] Low lipid levels can also exacerbate disease and contaminant loads, and can be a sign of poor overall fish condition. That said, hatchery steelhead, which are fed until release and likely have higher lipid levels, do not have higher early marine survival than wild steelhead (see study 1). Additional analyses are planned to assess whether the prevalence and intensity of *N. salmincola* affects lipid levels in steelhead smolts.

Juvenile steelhead size at outmigration and steelhead outmigrant abundance are not correlated with survival among years. Size at outmigration is also not correlated with survival within years – Early efforts associated with study 7 used available data describing fish characteristics to investigate correlations with steelhead SARs/marine survival trends. For the populations initially assessed, smolt weight, recorded for hatchery releases, showed no correlation with overall marine survival. However, data are limited to some and not all populations so a Puget Sound-wide assessment of these data could not be performed. This is consistent with early marine survival acoustic telemetry studies 1 and 8, where fork length was not correlated with early marine survival, showing no evidence of size selective mortality that would have derived in freshwater. However, a study of wild Skagit steelhead did conclude that size-selective mortality was occurring in the marine environment (Thompson and Beauchamp 2014).[24] It could be that size-selective mortality is a driver for open ocean survival of these wild steelhead. Initial efforts associated with study 7 also found no correlation between steelhead outmigrant abundance (smolt count/hatchery release number) and overall marine survival.

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[18] Sheridan, M.A., V. Allena ND, T.H. Kerstetter. 1983. Seasonal variations in the lipid composition of the steelhead trout, *Salmo gairdneri* Richardson, associated with the parr- smolt transformation. Journal of Fish Biology: 23, 125-134.

[19] Stefansson, B.T. Bjornsson, K. Sundell, G. Nyhammer, S.D. McCormick. 2003. Physiological characteristics of wild Atlantic salmon post-smolts during estuarine and coastal migration. Journal of Fish Biology. 63:942-955.

[20] Sheridan et. al. AND Stefansson et. al. (see above)

[21] Fessler, J.A. 1969. Some morphological and biochemical changes in steelhead trout during the parr-smolt transformation. Thesis. Oregon State University.

[22] McMillan, J.R., G.H. Reeves, C.E. Jordan. 2011. Individual condition and stream temperature influence on early maturation of rainbow and steelhead trout, Oncorhynchus mykiss. Environmental Biology of Fish. DOI 10.1007/s10641-011-9921-0

[23] Biro, P.A., A.E. Morton, J.R. Post, E.A. Parkinson. 2003. Over-winter lipid depletion and mortality of age-0 rainbow trout (Oncorhynchus mykiss). Canada Journal of Fisheries and Aquatic Sciences. **61**: 1513–1519.

[24] Thompson, J.N., and Beauchamp, D.A. 2014. Size-selective mortality of steelhead during freshwater and marine life stages related to freshwater growth in the Skagit River, Washington. Trans. Am. Fish. Soc. 143: 910–925.

Factors affecting predator-prey dynamics in the marine environment

An increase in the abundance of harbor seals correlates with the decline in steelhead. Abundance data are lacking for a correlative assessment of the other potential predators; however, qualitative information suggests there may be less of an association with the decline in steelhead survival – See the description of predators under Q2, above, for details.

The presence of alternative or "buffer" prey, in high abundance, may improve steelhead survival – Environmental factors can affect steelhead predation risk. As indicated previously, steelhead early marine survival more than doubled in 2016 and continued in 2017 compared to previous years' studied. Numerous sources suggest a significant increase in northern anchovy abundance in Puget Sound in 2016 that may have continued into 2017.[25] Study 5 discusses the association between increased steelhead early marine survival and the abundance of anchovies. Further, anchovy were identified in seal diets during the steelhead outmigration period (Study 6); however, low sample sizes limited any ability to make inferences regarding a buffer prey affect. Northern anchovy are energy-rich and school in nearshore areas in spring, summer and fall.[26] Steelhead do not typically swim near the shore and instead prefer the open waters of Puget Sound. If predators are redirected toward anchovies, it may lower predation on steelhead. A similar affect, but with juvenile rockfish, was found off the California Coast. Common murre consumption of out-migrating juvenile Chinook salmon increases (and survival declines) when murre distribution moves inshore, to feed on anchovies, vs offshore, to feed on rockfish.[27]

Long-term trends of other potential buffer prey were assessed for correlations with survival. Study 7 did find a positive correlation between the abundance of hatchery Chinook subyearlings released in Puget Sound and steelhead survival. More hatchery coho were also released during the period of higher steelhead survival; however, Chinook subyearlings have dominated salmon releases into Puget Sound and their release trend alone provided a stronger correlation with steelhead survival. Study 7 did not find a relationship between herring spawning abundance and steelhead survival. However, given how dynamic herring populations are, spawner abundance may not be the best metric for assessing their pelagic abundance during the steelhead outmigration period. The Workgroup will continue to investigate the buffer prey hypothesis.

A decline in the abundance of hatchery Chinook, combined with more consolidated release timing of hatchery Chinook subyearlings, may affect predator behavior and make steelhead more vulnerable to predation. Study 7 assessed multiple variables for correlations with steelhead marine survival since the 1970s. In addition to the strong inverse correlation with seal abundance, per the discussion above, there was a positive correlation between abundance of outmigrating hatchery Chinook and steelhead marine survival. Further, the CV (coefficient of variation) of hatchery subyearling Chinook release date had a positive, although more moderate, relationship with marine survival. Chinook subyearling release dates

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[25] Duguid, W. 2018. Historical fluctuations and recent observations of Northern Anchovy in the Salish Sea (manuscript submitted for publication).

[26] http://usa.oceana.org/responsible-fishing/northern-anchovy

[27] Wells, B.K, J.A. Santora, M.J. Henderson, P. Warzybok, J. Jahncke, R. W. Bradley, D. D. Huff, I.D. Schroeder, P. Nelson, J.C. Field, D.G. Ainley 2017. Environmental conditons and prey-switching by a seabird predator impacts juvenile salmon survival. *Journal of Marine Systems.* Vol 174: 54-63

have become consolidated since the 1980s. While a high abundance of highly distributed prey could buffer predation impacts to steelhead, declines in the number of hatchery fish released that are also becoming more consolidated could alter the behavior of predators. A pulse of fish could attract predators to specific places, at specific times, making steelhead more vulnerable to predation. A predator response to hatchery releases is well documented in Alaska.[28] Fish released from hatcheries may not immediately enter the Puget Sound marine environment; therefore, release data is not the best indicator for marine entry timing. Future work will focus on determining whether there is any alignment between hatchery Chinook entry timing into the Puget Sound marine environment and within-year early marine mortality patterns of steelhead.

The presence of transient killer whales may impact harbor seal and harbor porpoise behavior and abundance. Study 5 notes the increasing time transient killer whales are spending in Puget Sound. Transient killer whales consume harbor seals and harbor porpoises. The increased time they are spending in Puget Sound may be impacting the abundance harbor seals and harbor porpoise. Further, the presence during the steelhead outmigration period may affect the behavior of harbor seals and harbor porpoise. The Workgroup will investigate whether there is further support for this hypothesis.

Increased water clarity and light pollution may exacerbate predation; however, paucity of data limits analyses. Increased water clarity (reduced turbidity) is well documented to potentially lead to increased predator-prey encounter rates. However, its very predator dependent. [29] Recent data suggest water clarity is increasing in Puget Sound. However, the data are limited to the past twenty years and could not be included in the time series analysis (study 7). Further, an increase in light pollution can improve the efficacy of visual predators. Light pollution has increased globally over the past 30 years, with the time period of increase likely longer.[30] There has been no effort to compile local trend data on this phenomenon; however, Dr. Dave Beauchamp of US Geological Survey is currently using visual foraging models (VFM) for piscivorous, resident adult salmonids to map the nocturnal predation threat environment for juvenile salmon and steelhead in nearshore and offshore marine habitats within Puget Sound (affiliated work within the auspices of the Salish Sea Marine Survival Project).

Other environmental drivers including Puget Sound sea-surface temperatures and the North Pacific Index may contribute to the factors affecting overall marine survival. Study 7 noted that Puget Sound sea-surface temperatures and North Pacific Index values were negatively correlated with steelhead marine survival. Pacific Decadal Oscillation had a more moderate relationship with steelhead marine survival, with variable influence.

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[28] Chenoweth, E.M., J.M. Straley, M.V. McPhee, S. Atkinson, S. Reifenstuhl. 2017. Humpback whales feed on hatchery-released juvenile salmon. *Royal Society Open Science*. 4(7): 170180.

[29] Described in: Steelhead Marine Survival Workgroup. February 2014. Salish Sea Marine Survival Project - Research Work Plan: Marine Survival of Puget Sound Steelhead. Long Live the Kings, Seattle, WA. www.marinesurvivalproject.com.

[30] Li, X.; Zhou, Y. A Stepwise Calibration of Global DMSP/OLS Stable Nighttime Light Data (1992–2013). *Remote Sens.* 2017, *9*, 637

Next steps

The revised logic model, based upon the findings, is below. A crosswalk between the revised logic model and the findings is included as an appendix to this document. Funding is being sought for the third and final research phase of this effort. In the and next phase of research, the Workgroup will:

1) Continue to assess steelhead early marine survival rates, predation, and factors that may affect the extent of predation including Chinook hatchery release magnitude and timing, forage fish abundance, and presence/absence of transient whales.
2) Re-examine the extent to which the *N. salmincola* parasite leads directly or indirectly to mortality.
3) Identify *N. salmincola* hotspots in the Nisqually and Green rivers and recommend actions to reduce their loads.
4) Complete the work to isolate the sources of contaminants in the Nisqually River.

The Workgroup is also currently working with the Puget Sound Steelhead Recovery Team to incorporate their results in the Steelhead Recovery Plan. Here, management actions and continued monitoring and evaluation needs will be recommended.

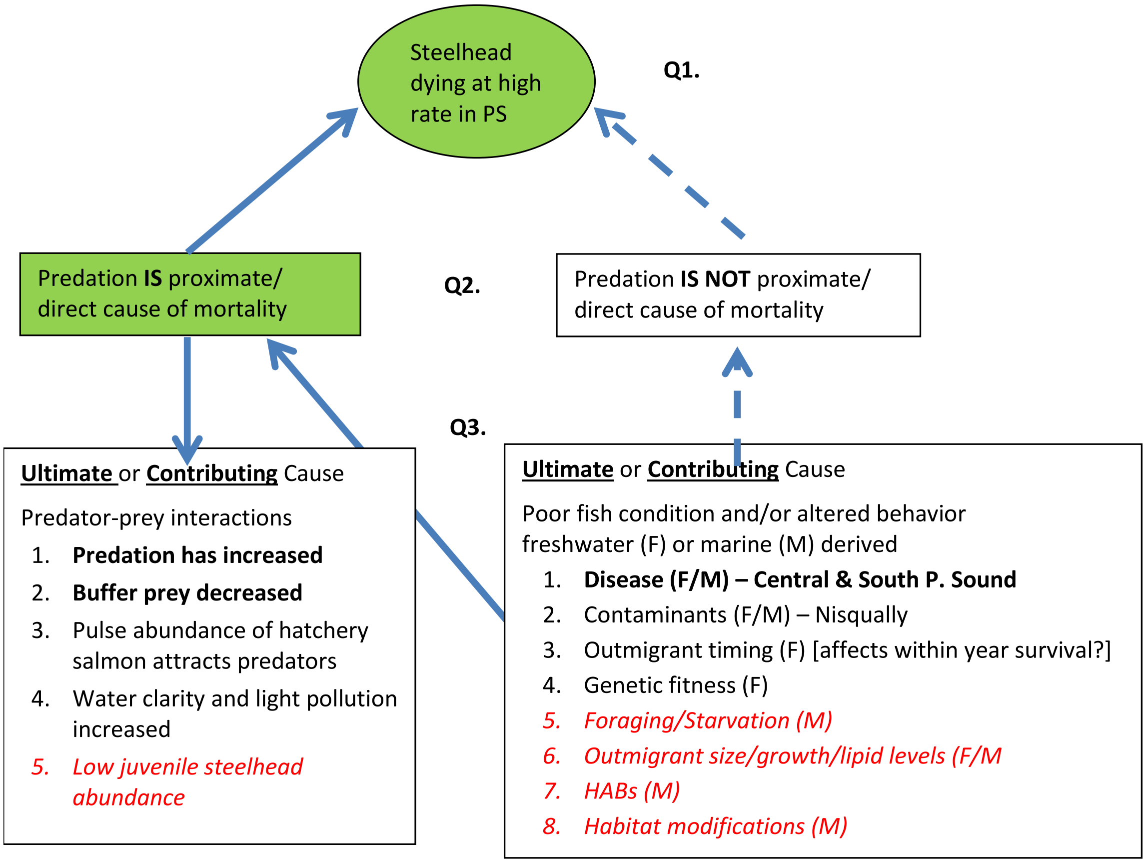

Figure 2. Updated Puget Sound steelhead marine survival evaluation. The factors are roughly ranked based upon existing evidence. Those in red have been found to be less likely to contribute to early marine mortality.

APPENDIX A: EXTENDED ABSTRACTS

Study 1: Multi-population analysis of Puget Sound steelhead survival and migration behavior

Megan E. Moore[1], Barry A. Berejikian[1], Frederick A. Goetz[2], Andrew G. Berger[3], Sayre S. Hodgson[4], Edward J. Connor[5] and Thomas P. Quinn[6]

[1]Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration Fisheries, P.O. Box 130, Manchester, 98353, USA

[2]U.S. Army Corps of Engineers, 4735 East Marginal Way South, Seattle WA 98134

[3]Puyallup Tribe Fisheries Department, Puyallup Tribe of Indians, 3009 East Portland Avenue, Tacoma WA 98404

[4]Department of Natural Resources, Nisqually Indian Tribe, 4820 She-Nah-Hum Drive, Olympia WA 98513

[5]Seattle City Light, City of Seattle, 700 Fifth Ave. Seattle WA 98104, USA

[6]School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle, WA 98195

Until recently, research on mortality of anadromous fishes in the marine environment was largely limited to estimates of total mortality and association with group characteristics or the environment. Advances in sonic transmitter technology now allow estimates of survival in discrete marine habitats, yielding important information on species of conservation concern. Previous telemetry studies of steelhead Oncorhynchus mykiss smolts in Puget Sound, Washington, USA indicated that approx. 80% of fish entering marine waters did not survive to the Pacific Ocean. The present study re-examined data from previous research and incorporated data from additional Puget Sound populations (n = 7 wild and 6 hatchery populations) tagged during the same period (2006–2009) for a comprehensive analysis of steelhead early marine survival. We used mark-recapture models to examine the effects of several factors on smolt survival and to identify areas of Puget Sound where mortality rates were highest. Wild smolts had higher survival probabilities in general than hatchery smolts, with exceptions, and wild smolts released in early April and late May had a higher probability of survival than those released in early and mid-May. Steelhead smolts suffered greater instantaneous mortality rates in the central region of Puget Sound and from the north end of Hood Canal through Admiralty Inlet than in other monitored migration segments. Early marine survival rates were low (16.0 and 11.4% for wild and hatchery populations, respectively) and consistent among wild populations, indicating a common rather than watershed-specific mortality source. With segment-specific survival information we can begin to identify locations associated with high rates of mortality, and identify the mechanisms responsible.

(Publication: Moore, M.E., B.A. Berejikian, F.A. Goetz, A.G. Berger, S.S. Hodgson, E.J. Connor, T.P. Quinn. 2015. Multi-population analysis of Puget Sound steelhead survival and migration behavior. Marine Ecology Progress Series, 537 (217-232). DOI: 10.3354/meps11460.)

Study 2: Declining patterns of Pacific Northwest steelhead trout (*Oncorhynchus mykiss*) adult abundance and smolt survival in the ocean

Neala W. Kendall, Gary Marston, and Matthew Klungle

Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501-1091 USA

Examination of population abundance and survival trends over space and time can guide management and conservation actions with information about the spatial and temporal scale of factors affecting them. Here, we analyzed steelhead trout (anadromous Oncorhynchus mykiss) adult abundance time series from 35 coastal British Columbia and Washington populations along with smolt-to-adult return (smolt survival) time series from 48 populations from Washington, Oregon, and the Keogh River in British Columbia. Over 80% of the populations have declined in abundance since 1980. A multivariate autoregressive statespace model revealed smolt survival four groupings: Washington and Oregon coast, lower Columbia River, Strait of Juan de Fuca, and Puget Sound – Keogh River populations. Declines in smolt survival rates were seen for three of the four groupings. Puget Sound and Keogh River populations have experienced low rates since the early 1990s. Correlations between population pairs' time series and distance apart illustrated that smolt survival rates were more positively correlated for proximate populations, suggesting that important processes, including those related to ocean survival, occur early in the marine life of steelhead.

(Publication: Kendall, N.W., G.W. Marston, and M.M. Klungle. 2017. Declining patterns of Pacific Northwest steelhead trout (Oncorhynchus mykiss) adult abundance and smolt survival in the ocean. Can. J. Fish. Aquat. Sci. 74: 1275–1290 (2017) dx.doi.org/10.1139/cjfas-2016-0486.)

Study 3: Identifying potential juvenile steelhead predators in the marine waters of the Salish Sea

Scott F. Pearson[1], Steven J. Jeffries[1], Monique M. Lance[1], and Austen Thomas[2,3]

[1]Wildlife Science Division, Washington Department of Fish and Wildlife, Olympia

[2]Zoology Department, University of British Columbia, Vancouver

[3]Molecular Genetics Laboratory, Washington Department of Fish and Wildlife, Olympia

(Technical Report: Pearson, S.F., S.J. Jeffries, M.M. Lance and A.C. Thomas. 2015. Identifying potential juvenile steelhead predators in the marine waters of the Salish Sea. Washington Department of Fish and Wildlife, Wildlife Science Division, Olympia. Available at www.marinesurvivalproject.com/resources.)

Puget Sound wild steelhead were listed as threatened under the Endangered Species Act in 2007 and their populations are now less than 10% of their historic size. Data suggest that juvenile steelhead mortality is very high in the marine waters of the Salish Sea (waters of Puget Sound, the Strait of Juan de Fuca and the San Juan Islands as well as the waters surrounding British Columbia's Gulf Islands and the Strait of Georgia). Understanding the mechanism(s) responsible for low steelhead survival in the Salish Sea can help inform potential management solutions. One potential mechanism is top-down predation by fish-eating predators. To help us better understand the potential role of predators in steelhead decline, we identified possible marine mammal and bird predators of outmigrating juvenile steelhead based on predator distribution, abundance, and diet information. Given this review, we identified the next steps (research and information needs) for identifying and evaluating predation as a potential mechanism for low early marine steelhead survival.

Based on our literature review, we recommend that future research on the juvenile steelhead "predation hypothesis" focus on the diet, distribution and abundance of harbor seals, double-crested cormorants, Caspian terns, and Brandt's cormorants. In addition, although juvenile salmon have not been detected in stomach contents in Puget Sound, harbor porpoises have increased dramatically during the period of steelhead decline and, because they find their prey using echolocation, have a unique ability to exploit a resource like juvenile steelhead that tend to move individually or in small groups rather than in large schools. Finally, if additional resources are available, we would also include California sea lions and common murres. We recommend that research on this suite of potential predators be focused on gaining a better understanding of predator space use, foraging areas, and diet composition in areas of apparently high juvenile steelhead mortality (Hood Canal bridge area, Admiralty Inlet, and Central Puget Sound). All of these fish-eating species identified for additional research have demonstrated relatively stable or increasing population trends in recent years and their diet includes juvenile salmon, even if only a very minor component. To help us narrow the list of potential predators, we recommend initial surveys to assess relative predator abundance in areas of high steelhead mortality during the steelhead outmigration window – a period when we have poor information on predator abundance and distribution. One approach for assessing predator diet is to use new molecular techniques in combination with traditional techniques (hard part analysis) to help us understand the importance of steelhead to predator diet. This multiple predator approach has advantages in that it may not be a single predator that is contributing to low steelhead survival. If predation is identified as a factor contributing to steelhead declines, it is also important to gain a better understanding of potential ultimate factors that may be leading to high predation rates such as steelhead physical condition, potential hatchery effects, and human environmental modifications such as the Hood Canal Bridge.

Table 1. Mammals and birds from Gaydos and Pearson (2011) that are relatively abundant in central and northern Puget Sound in the spring and summer and are fish eaters (piscivorous). We reviewed the literature to assess: 1) the degree of size overlap between fish in the diet and the size of outmigrating steelhead, 2) any evidence that the predator eats juvenile salmon and/or steelhead, and 3) and evidence that the predator eats juvenile steelhead. The species highlighted in green eat fish the size of outmigrating steelhead.

Common name	Scientific name	Diet overlap[1]	Eat Juvenile salmon or steelhead?[2]	Eat Juvenile steelhead?[2]
Mammals				
Harbor porpoise	*Phocoena phocoena*	Yes	No evidence	No evidence
Dall's porpoise	*Phocoenoides dalli*	Yes	No evidence	No evidence
Harbor seal	*Phoca vitulina*	Yes	Yes	Yes
California sea lion	*Zalophus californianus*	Yes	Yes	Yes
Birds				
Common loon	*Gavia immer*	Yes	?	?
Pacific loon	*Gavia pacifica*	Likely	Yes	?
Red-throated loon	*Gavia stellata*	Yes	?	?
Western grebe	*Aechmophorus occidentalis*	No	?	?
Red-necked grebe	*Podiceps grisegena*	Little	?	?
Horned grebe	*Podiceps auritus*	No	?	?
Double-crested cormorant	*Phalacrocorax auritus*	Yes	Yes	Yes (no local evidence)
Brandt's cormorant	*Phalacrocorax penicillatus*	Yes	Yes	?
Pelagic cormorant	*Phalacrocorax pelagicus*	Yes	?	?
Red-breasted merganser	*Mergus serrator*	Unlikely	Yes	?
Glaucous-winged/Western gull complex	*Larus glaucescens, L. occidentalis, and L. glaucescens x L. occidentalis*	Likely	Yes	Yes (no local evidence)
Caspian tern	*Sterna caspia*	Yes	Yes (estuary)	Yes
Common murre	*Uria aalge*	Moderate	Yes	?
Rhinoceros auklet	*Cerorhinca monocerata*	Little	Yes	No evidence
Pigeon guillemot	*Cepphus columba*	Little	No evidence	No evidence
Marbled murrelet	*Brachyramphus marmoratus*	No	Yes (freshwater)	?

[1] Yes = literature indicates that the predator regularly eats fish the size of juvenile steelhead; No = only eats fish smaller that juvenile steelhead; likely = little or no information on fish length in diet but based on the size of fish consumed by a similar sized congeneric, it is likely that they eat appropriate sized fish; Little = only the longest fish consumed overlap with the smallest juvenile steelhead; Moderate = approximately half of the fish consumed are similar to small to moderately sized juvenile steelhead.

[2] Yes = the literature indicates that they eat juvenile salmon and or steelhead; Yes (no local evidence) = documented to eat steelhead but there is no evidence from the Salish Sea despite considerable diet samples; No evidence = despite large sample sizes in the literature (100s of samples), there is no evidence that the species eats salmon/steelhead; ? = data are not adequate to evaluate this question.

Study 4: Predator-prey interactions between harbor seals and migrating steelhead smolts revealed by acoustic telemetry

B. A. Berejikian[1], M. E. Moore[1], and S. J. Jeffries[2]

[1]Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, NOAA, 7305 Beach Drive East, Port Orchard, WA 98366

[2]Washington Department of Fish and Wildlife, 600 Capitol Way North, Olympia, WA 9850

Changes in the Puget Sound ecosystem over the past 3 decades include increases in harbor seal (*Phoca vitulina*) abundance and declines in many of their preferred prey species. Harbor seals were outfitted with acoustic telemetry receivers and GPS tags to investigate spatial and temporal interactions with steelhead trout Oncorhynchus mykiss smolts implanted with acoustic transmitters. A total of 6846 tag detections from 44 different steelhead trout smolts (from an initial group of 246 smolts released into 2 rivers) were recorded by the 11 recovered sealmounted receivers. Central Puget Sound seal receivers detected a greater proportion of smolts surviving to the vicinity of the haul-out locations (29 of 51; 58%) than Admiralty Inlet seal receivers (7 of 50; 14%; $p < 0.001$). Detection data suggest that none of the tagged smolts were consumed by the 11 monitored seals. Nine smolts were likely consumed by non-tagged harbor seals based partly on detections of stationary tags at the seal capture haul-outs, although tag deposition by other predators cannot be ruled out. Smolts implanted with continuously pinging tags and smolts implanted with tags that were silent for the first 10 d after release were detected in similar proportions leaving Puget Sound (95% CI for the difference between proportions: −0.105 to 0.077) and stationary at harbor seal haul-outs (95% CI: −0.073 to 0.080). This study suggests that harbor seals contribute to mortality of migrating steelhead smolts, and we hypothesize that documented changes in the Puget Sound ecosystem may currently put steelhead smolts at greater risk of predation by harbor seals and possibly other predators.

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Study 5. Interactions between harbor seals and steelhead in Puget Sound, and phase 2 of assessing tag noise effects on survival

Berejikian, B.A.[1,]M. E. Moore[1], and S. J. Jeffries[2]

[1] Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, NOAA, Manchester Research Station, 7305 Beach Drive East, Port Orchard, WA 98366

[2] Washington Department of Fish and Wildlife, 600 Capitol Way North, Olympia, WA 9850

This study investigated the migratory behavior and survival of acoustic-tagged Nisqually River steelhead smolts in Puget Sound. Previous work has indicated that steelhead smolts migrating through Puget Sound experience high mortality rates (Moore et al. 2015), mortality is most acute in Central Puget Sound (Tacoma Narrows to Jefferson Head; Moore and Berejikian 2017), harbor seals are predating on smolts (Berejikian et al 2016), and the noise associated with acoustic tags has no effect on survival or inferred predation by harbor seals (Berejikian et al. 2016). The study was designed on the basic assumption that the chronically low marine survival of Nisqually River steelhead smolts documented from 2006-2009 and again in 2014 would continue in 2016. The objective was to increase tag detection capacity and follow on the 2014 work to determine from tag detection patterns the fractions of Nisqually River steelhead smolts that i) survive migration through Puget Sound, ii) are consumed by harbor seals, iii) exhibit other patterns.

Methods

Steelhead smolts from the Nisqually River (N=199) were surgically implanted with Vemco V7 69 kHz acoustic transmitters over a period of 6 weeks (23 April to 2 June) and released at river-kilometer 20. Fifty of the 199 transmitters were programmed to turn on 10 days after tagging to test whether smolts tagged with actively pinging ('continuous tags') transmitters survived at a lower rate than those with transmitters that were silent for the majority of the Puget Sound migration and subsequently switched on so they could be detected at outer arrays (i.e., 'delay tags').

Three sampling platforms were used to describe movement patterns, estimate survival rates, infer predation events, and determine the fates of individual steelhead: 1) mobile hydrophones mounted on harbor seals, 2) fixed hydrophones moored in the Nisqually estuary and Puget Sound (Figure 1), and 3) boat-mounted mobile tracking. Fixed hydrophones were moored on the seafloor to detect smolts as they migrated past the Nisqually river mouth (RM), the Tacoma Narrows (NAR), Central Puget Sound (CPS), Admiralty Inlet (ADM), and finally the Strait of Juan de Fuca (JDF; Figure 1). Cormack-Jolly-Seber survival models (Lebreton et al. 1992) were used to estimate apparent survival and detection probabilities of tagged smolts at each hydrophone array. Mobile hydrophones were mounted on sixteen harbor seals captured at Puget Sound haulout sites in North Puget Sound (Colvos Rocks area, N = 4), Central Puget Sound (Orchard Rocks, N = 4), and South Puget Sound (Gertrude Island, N = 1; Nisqually estuary, N = 3, Eagle Island, N = 4). Each instrument pack contained a Vemco VMT receiver capable of detecting the V7 tags, a satellite-linked time depth recorder (TDR) and Fastloc GPS tag (model MK10AF, Wildlife Computers, www.wildlifecomputers.com), and a VHF tag (164−165 MHz, Advanced Telemetry Systems; www.atstrack.com) used for locating the instrument packs after they had been shed by the harbor seals (Berejikian et al. 2016). A boat-deployed (mobile) hydrophone was used to detect tags at pre-determined locations in nearshore habitats after smolts completed migration (Figure 2).

Tag noise has no effect on survival

To compare the survival of delay and continuous tags detected at the stationary arrays in Puget Sound, only continuous tags that were detected at least 10 days after tagging were considered. In this way the survival comparison for delay and continuous tags was unbiased. The proportion of delay-tagged smolts detected at each of the stationary arrays in Puget Sound was similar to the expected proportion 25% (Table 1). At the final array located in the Strait of Juan de Fuca, 21% percent of the tags detected after 10 d of activation were delay tags.

In 2014, the 95% confidence interval for the difference between the proportions (continuous - delay) was -0.105 to 0.077, indicating that the two tag types were significantly not different from one another (two-tailed test). A significant difference would have been indicated by 95% confidence intervals that did not include 0.

In 2016, the continuous tags were detected as surviving to the JDF array in a slightly higher than expected proportion (Table 1). The two-tail 95% confidence interval for the difference between the proportions (continuous - delay) was -0.0794 to 0.1484, and the power was estimated at 0.89 with a 0.15 tolerance limit (i.e., the critical difference between proportions). Thus, in both years, the proportions were significantly not different, and in 2016 there was high power to detect a 15% difference in survival

Preliminary results

Nisqually steelhead smolts survived the migration from river mouth to the Strait of Juan de Fuca at a much higher probability (38%) in 2016 than in previous years (Figure 2). Estimated survival probabilities in 2016 were particularly high through the NAR-CPS (85.6 ± 5.6%) and CPS-ADM (80.6 ± 7.4%) migration segments, especially in comparison to survival estimates through the same segments in 2014 (NAR-CPS: 33.8 ± 6.9%; CPS-ADM: 55.5 ± 8.5%). The mark-recapture survival estimates from the continuous tags were applied to both the delay and continuous tag types to estimate the total number of tagged smolts reaching the Nisqually estuary and each of the main arrays. An estimated 139 of the 199 smolts survived to the Nisqually River estuary, 98 survived to the NAR array, 83 to the CPS array, 68 to the ADM array, and 53 to the JDF array (Figure 3).

The 15 recovered seal mounted VMT hydrophones detected 78 different steelhead smolts a total of 6,585 times (mean = 84 detections; range = 1-1,128). Detections of steelhead tags by harbor seals occurred both during and after the spring smolt outmigration period (the last smolt was detected at JDF on 28 June 2016). VMT-detection locations were determined for 58 of the 78 detected smolts (range = 1 – 784 per smolt), based on close time associations (<15 min) between the tag detection and a Fastloc GPS location for the detecting seal.

In 2016, the fates of the 199 tagged steelhead smolts were categorized based on their movement patterns and final detection location. One hundred, twenty-five smolts were detected by at least one of the three detection methods in the estuary or further along their migration path. No tags were determined to be stationary at or near the Orchard Rocks, Eagle Island, Gertrude Island harbor seal haulouts. One tag was stationary near the Colvos Rocks haulout. Two tags were detected as stationary near the Point Defiance haulout, where instrumented seals spent considerable time during the spring and summer. However, 16 tags were determined to be stationary within 4 km of the Nisqually estuary seal tagging location (figure 3). Of these 16 tags, 11 exhibited back-and-forth movements in the Nisqually River and estuary consistent with harbor seal movements (upstream at higher tides and downstream at lower tides; Moore and Berejikian 2017). An additional four tags were detected simultaneously moving back and forth through the river and estuary, and all four were detected stationary in the same location 9 km NE of the haulout near Ketron Island. The spatial distribution of survivors, stationary transmitters, and smolts with unknown fates throughout Puget Sound is shown in figure 3. By comparison, in 2014, 9 steelhead smolt transmitters were detected stationary tags at the Colvos Rocks, Orchard/Blakely Rocks seal capture haulouts. In 2014, study did not include monitoring of the Nisqually estuary where most of the stationary tag detections occurred in 2016. Additional analyses will include hierarchical Bayesian occupancy models to assess predation risk in both 2014 and 2016.

Summary and next steps

There were substantial differences in survival and evidence of predation by harbor seals compared with the initial investigation in 2014. In 2014, mark-recapture estimates indicated that survival of steelhead through Central Puget Sound (Tacoma Narrows to Admiralty Inlet) was low (19%) and stationary tags were detected at harbor seal haulouts. In 2016 survival of steelhead through Central Puget Sound was high (69%), and no steelhead tags were detected stationary at harbor seal haulouts in the same region. However, in 2016, evidence of predation by harbor seals increased in the Nisqually estuary. Further, in both years, detection patterns of some tags were consistent with harbor seal movements, suggesting that tagged smolts had been eaten and were being carried by harbor seals. Steelhead smolt migratory behavior patterns through the Puget Sound epi-pelagic environment were very similar in the two years, and therefore, do not likely explain the differences in survival or predation risk. Over the course of this study, there have been major events that may be related the observed shifts in predation risk. First, transient killer whale frequency has increased in recent years, and that may have an impact on mammalian predators such as harbor seals and harbor porpoise. Second, a major pulse of anchovies occurred in 2016, and may have continued into 2017. This may have provided providing abundant alternative prey for predators of steelhead smolts. Both of these events are consistent with the increased survival of steelhead smolts, and we are currently evaluating data to determine the strengths of these relationships.

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Table 1. Detections of delay and continuous acoustic transmitters at each of the fixed arrays in Puget Sound. Delay tags first began emitting acoustic pings 10 days after tagging. Therefore, to produce an unbiased comparison at each line, only continuous tags detected after 10 days were considered.

	Detections after 10 days			
	NAR	CPS	ADM	JDF
Continuous	14	23	22	26
Delay	5	8	13	7
Delay %	26%	26%	37%	21%
Expected%	25%	25%	25%	25%

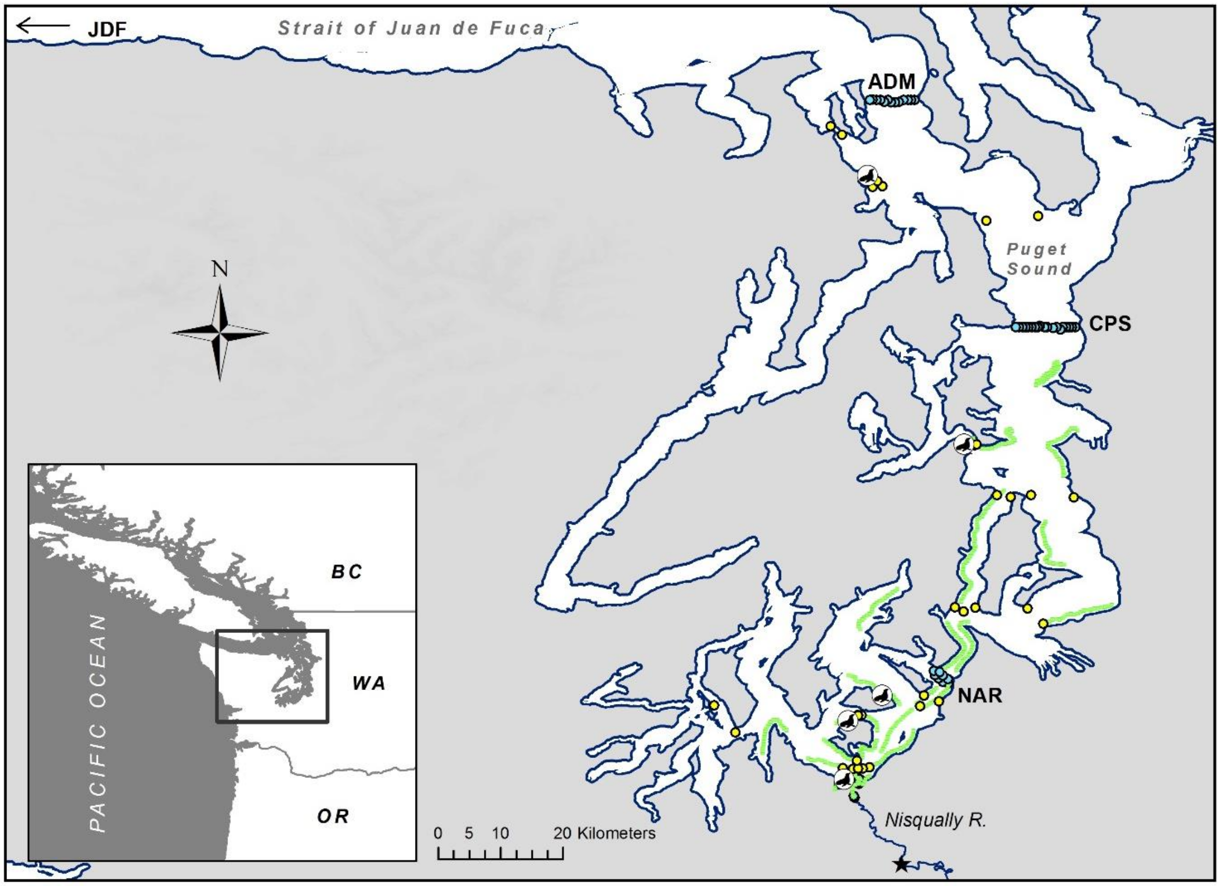


Figure 1. Locations of fixed Vemco VR2 (yellow circles) and VR3/4 (blue circles) hydrophones in the Nisqually estuary and Puget Sound. Areas covered by mobile tracking are indicated by green squares, and seal tagging locations are marked by a seal icon. The black star shows where tagged steelhead smolts were released at rkm 20.

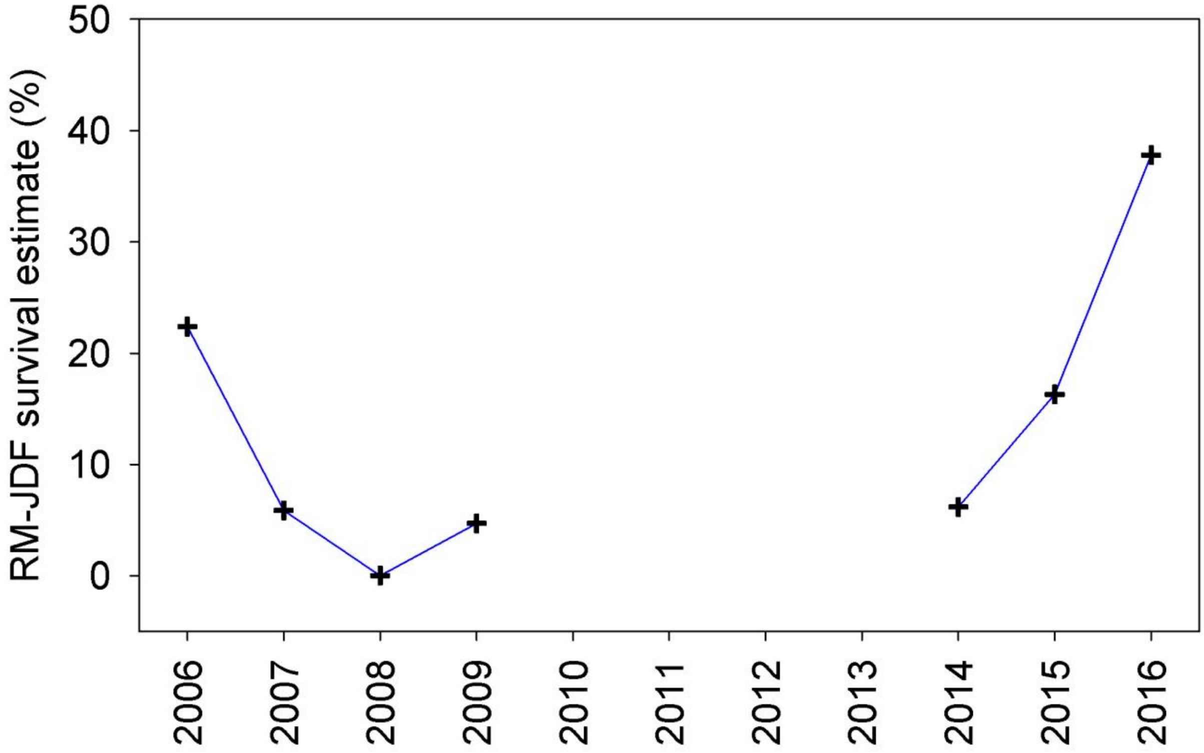


Figure 2. Nisqually River smolt survival probabilities, from river mouth (RM) to Strait of Juan de Fuca (JDF). No studies were conducted between 2010 and 2013.

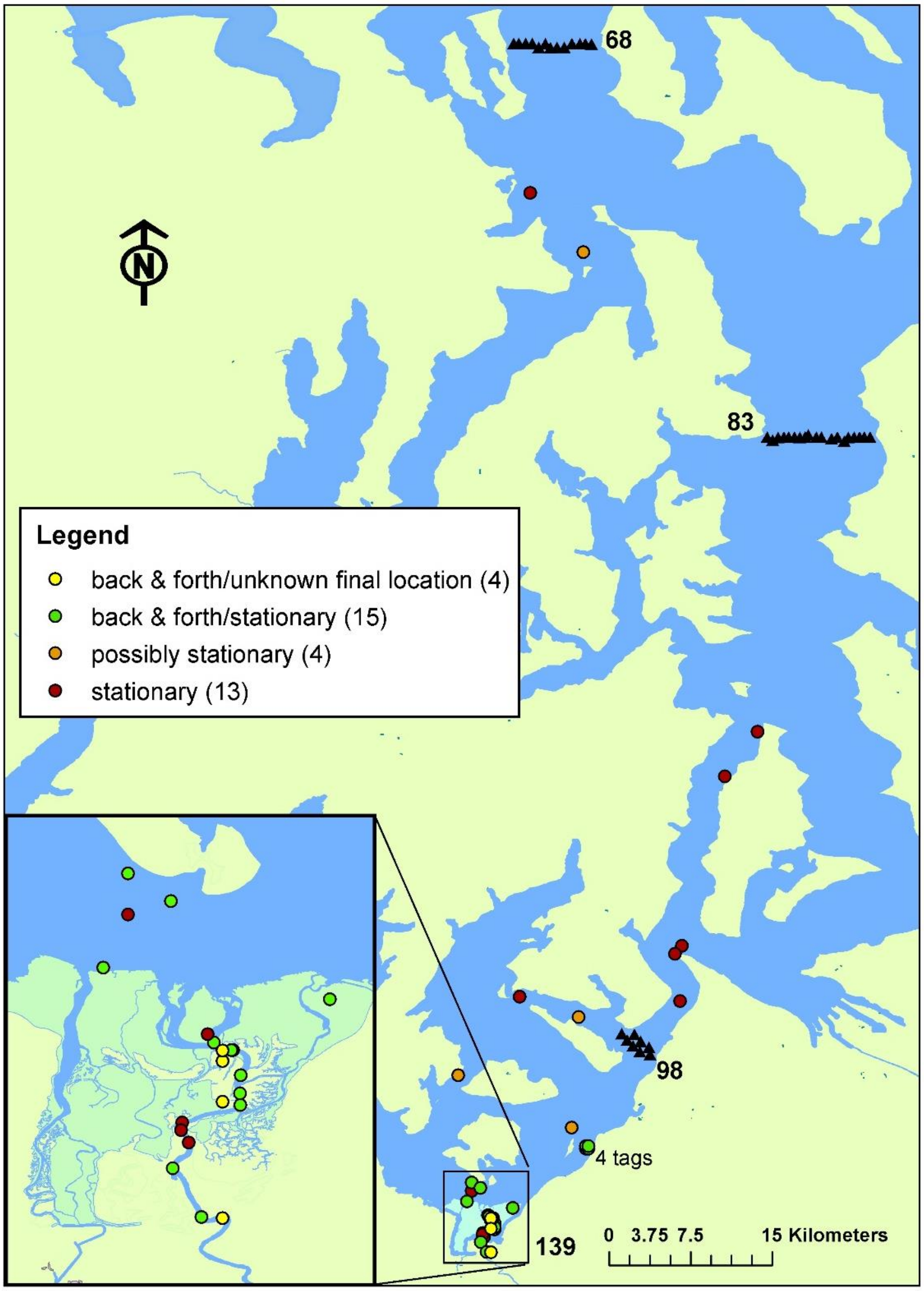


Figure 3. Fates of all steelhead smolts detected at least as far as the Nisqually estuary. Red dots show where transmitters were found to be stationary based on number and timing of detections. Orange dots depict locations where detection timing and location suggest a stationary disposition, but evidence is not conclusive. Green dots indicate transmitters detected as stationary that previously moved repeatedly back and forth from upper to lower Nisqually river estuary or Nisqually Reach, and yellow dots indicate transmitters with back and forth behavior that were not detected as stationary, but did not survive to any further arrays. Black numbers represent the number of smolts estimated to have survived to each adjacent receiver array based on mark-recapture survival probabilities.

Study 6: Puget Sound Harbor Seal Diet: Research Progress Update

Scott Pearson[1], Steven Jeffries[1], Austen Thomas[2], Monique Lance[1], and Amelia Louden[1]

[1]Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501
[2] Smith-Root Inc., 16603 NE 50th Avenue, Vancouver, WA 98686, USA

Introduction

Harbor seal predation is one of the hypothesized mechanisms responsible for low juvenile steelhead survival in Puget Sound during smolt outmigration (Pearson et al. 2015). Acoustic telemetry work conducted in Puget Sound and Hood Canal has produced indirect evidence of predation on juvenile steelhead by seals in Puget Sound (Berejikian et al. 2016, Moore and Berejikian 2017). However, we are currently lacking direct evidence of juvenile steelhead predation by harbor seals during the critical smolt outmigration period in the spring, or the information needed to quantify the impact of seal predation on juvenile salmonid including steelhead survival (Berejikian et al. 2016, Moore and Berejikian 2017).

In this study, we attempted to directly quantify seal consumption of juvenile salmonids with the focus on steelhead by examining seal diet from fecal samples (scats) using both prey hard part remains (e.g. Lance et al. 2012) and prey DNA (Thomas et al. 2016). To accomplish this, harbor seal scat samples were collected from seal haulouts in Puget Sound (Figure 1) in 2016. In addition, we estimate the proportional steelhead contribution to harbor seal population diet using the percentage of steelhead DNA contained in seal scat samples.

Specific study objectives:

- Quantify spring harbor seal diet using both hard parts and DNA
- Evaluate the impact of harbor seals on juvenile steelhead and salmonids

Methods & background

Study area, haulouts & scat collection.— Harbor seal scats were collected approximately every other week between 11 January and 22 August, 2016 from six seal haulout sites in southern Puget Sound (Figure 1). We sampled from 1) intertidal haulouts e.g. Gertrude Island and Eagle Island, primarily during low-tide conditions when we were more likely to be successful finding scats and 2) sites available at all tides e.g. Woodard Bay, Cutts Island and Commencement Bay, where scats are on log booms or high on the beach and not washed away by tides. The field crew attempted to collect a minimum of 70 harbor seal scat samples from all seal haulouts per collection windows, but our goal was sample sizes greater than 94 per month.

These sample sizes are needed because it is not uncommon to have > 35 species of prey being consumed by pinnipeds in a given locality, but only 3-15 of those species are typically common (≥5% of total diet) (Trites and Joy 2005). Trites and Joy (2005) recommended a sample size of at least 59 scats to identify principal prey occurring in >5% of scats. Additionally, they recommended a sample of 94 to compare moderate effects over time or between areas (Trites and Joy 2005). This sample size is a rule of thumb determined from a statistical power analysis for seal and sea lion diet studies. Because our goal was to identify dietary items that usually occur < 5% of the samples, we attempted to collect even larger sample sizes. Rarely detected species may have been consumed opportunistically (i.e., not targeted), or may even represent "secondary prey" which is when a prey species is in the stomach or gut of the prey that was eaten by the seal. In other words, these are prey not directly killed or targeted by the seal, but have hard parts or DNA that shows up in scats when processed because they were also consumed.

Scats can be used to examine pinniped diet in relationship to a foraging bout or meal and to calculate bioenergentics requirements. Based on captive feeding studies, a single foraging bout or "meal" occurs in 3.8 ± 1.8 scats [range 1–10, passed over 24-48 hour time period (Phillips and Harvey 2009)]. Each scat contains digested/degraded hard parts and DNA from a previous meal (or meals). As a result, we can use prey remains in scats to examine diet composition for studies focused on community level variation in diet, seasonal variability, geographic variability, and/or variability among haulout sites (Boyle *et al.* 1990, Cottrell *et al.* 1996, Brown and Pierce 1998, Cottrell and Trites 2002, Tollit *et al.* 2004). In addition, prey remains found in scats can be used to identify specific prey items of interest (Ward et al. 2012).

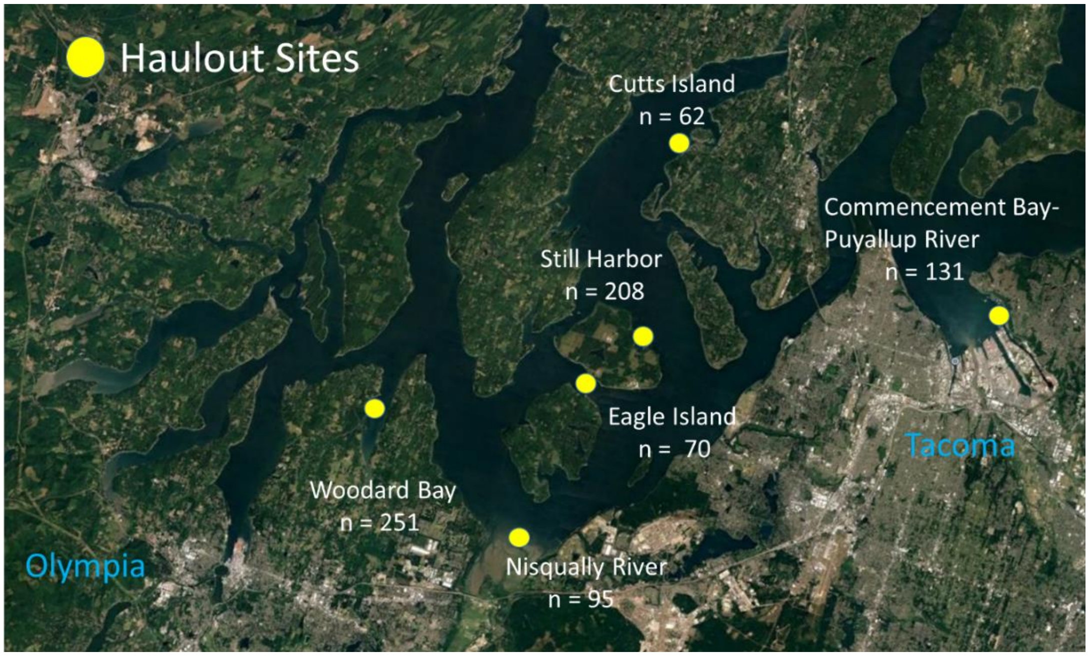


Figure 1. Location of harbor seal haulouts where scats were collected in Puget Sound, Washington with n = number of scats collected at each haulout. Note: Collection localities are well spaced geographically and represent estuarine (Commencement Bay and Nisqually River) and non-estuarine (Woodard Bay, Eagle Island, Still Harbor and Cutts Island) haulout sites used by harbor seals in Puget Sound.

Scat collecting and processing.—At the haulout sites, individual scat samples were collected with single use disposable gloves and wooden tongue depressor into a 126µm nylon mesh paint strainer bag inside of a one gallon Ziploc type plastic bag (Orr *et al.* 2003). Samples were taken to the lab and frozen at -20°C within 6 hours of collection (King *et al.* 2008).

For DNA extraction, each sample was thawed and depending on size, placed in 500 ml or 1000 ml plastic jar filled with ethanol. Each individual scat still inside the paint strainer bag, was homogenized manually into the ethanol with a disposable tongue depressor to separate the scat matrix material for DNA from hard part remains (e.g. otoliths, bones, cephalopod beaks). The paint strainer bag containing prey hard parts was then removed from the jar leaving behind the ethanol preserved scat matrix for genetic analysis (Thomas *et al.* 2014).

For hard parts sorting, remaining scat sample still enclosed in individual paint strainer bags were cleaned using a washing machine to remove organic material and retain prey hard parts (Orr *et al.* 2003). Scats collected from haulout sites like Eagle Island or Cutts Island, which have coarse sand or cobble rock substrate, nested sieves were used to separate rocks and other materials to preserve the integrity of the hard parts for identification (Lance et al. 2001). Hard parts were cleaned (i.e. flesh removed) and stored dry. Cephalopod beaks and cartilaginous parts were stored in isopropyl alcohol to prevent distortion for subsequent identification and measuring. Prey were identified to the lowest possible taxon using a dissecting microscope, reference fish bone collections from Washington and Oregon, and published fish bone, otolith and cephalopod beak keys (Morrow 1979, Wolff 1982, Clarke 1986, Cannon 1987, Harvey *et al*. 2000). Otoliths were measured using an ocular micrometer and graded based on observed erosion (Tollit *et al.* 1997, Tollit *et al*. 2004). We present data as percent frequency of occurrence (FO) that was standardized to total 100% per sample to convert the results to a comparable scale as the DNA results. We created species groups when presenting frequency of occurrence data to simplify and illustrate overall composition (e.g. “All Gadids” includes Pacific hake, Pacific tomcod, and Walleye Pollock).

Defining juveniles and adults.— Vertebrae diameters from the largest adult and juvenile salmon vertebrae found in a sample were measured to the nearest 1/10 mm and were used to define juveniles and adults using the natural break in diameter exhibited by the data (Figure 2).

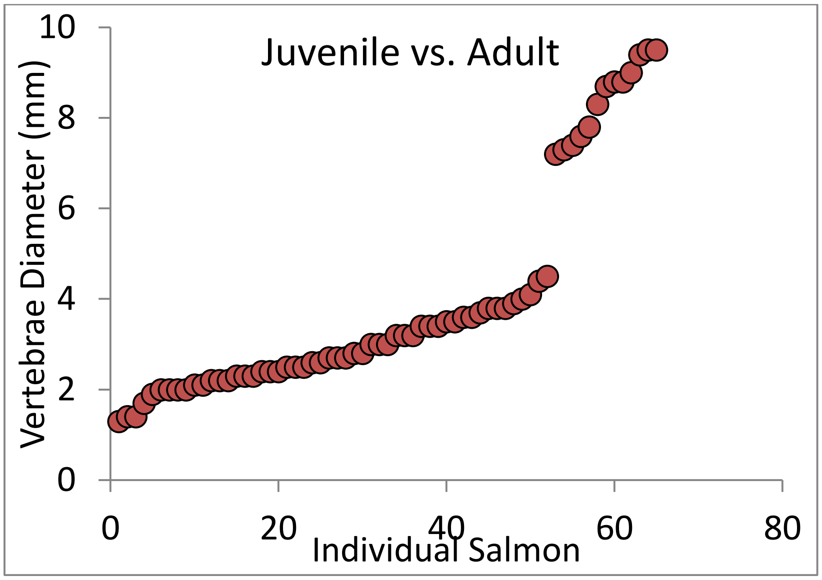


Figure 2. Break in vertebrae diameter measurements used to distinguish “juvenile” (< 4.5 mm) and “adult” (> 7.2 mm) salmon.

DNA reconstruction.-- The DNA metabarcoding marker used to quantify fish proportions from seal scats is a 16S mDNA fragment (~ 260 bp) previously described for pinniped scat analysis (Deagle et al. 2009). We used the combined Chord/Ceph primer sets: Chord_16S_F (GATCGAGAAGACCCTRTGGAGCT), Chord_16S_R (GGATTGCGCTGTTATCCCT), Ceph_16S_F (GACGAGAAGACCCTAWTGAGCT), and Ceph_16S_R (AAATTACGCTGTTATCCCT). This multiplex PCR reaction is designed to amplify both chordate and cephalopod prey species DNA. To ensure accurate salmon species identification, a secondary metabarcoding marker was used to quantity the salmon portion of seal diet, because the primary 16S marker is unable to differentiate between coho (*Oncorhynchus kisutch*) and steelhead (*Oncorhynchus mykiss*) DNA sequences in some cases. This marker is a COI "minibarcode" specifically for salmonids within the standard COI barcoding region: Sal_COI_F (CTCTATTTAGTATTTGGTGCCTGAG), Sal_COI_R (GAGTCAGAAGCTTATGTTRTTTATTCG). The COI amplicons were sequenced alongside 16S such that the overall salmonid fraction of the diet was quantified by 16S, and the salmon species proportions within that fraction was quantified by COI. All primers were indexed with unique 10pb tags on both the forward and reverse oligos to assist in demultiplexing and to avoid potential sample-to-sample contamination during the sequencing library prep process (Schnell et al. 2015). Amplicon sequencing was performed on an Illumina MiSeq, and FASTQ files were processed using a custom bioinformatics pipeline for pinniped scat analysis (Thomas et al. 2015). Harbor seal population diet percentages were then calculated from the DNA sequence percentages of all individual samples in a collection period - where the seal population diet percentage for a particular prey species represents the average species DNA sequences % calculated from all samples in a collection stratum (Thomas *et al.* 2016).

Results

We collected 832 scat samples, with most months having more than 82 samples (Table 1). Note that six of the eight months had sample sizes > 90 and the critical months for steelhead outmigration of April and May had sample sizes greater than our target sample size. Because of the small sample size in June and because samples were only collected in early June, we combined the May and June samples for all data summaries that follow.

Table 1. Harbor Seal scat sample sizes by month and haulout. Note that sample sizes may be slightly smaller for the DNA analysis because some samples did not produce adequate DNA for analysis.

Site	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Total
Clam Bay[1]				1					1
Commencement Bay							64	53	117
Colvos Rocks[2]				1					1
Cutts Island	18	6	3		30	2	4		63
Eagle Island	14	29	9	2	11		3	2	70
Nisqually	13	21	19	1	15				69
Orchard Rocks[3]				1					1
Still Harbor	20	27	8	67	51	2	40	8	223
Woodard Bay	90	66	43	3	47	5	5	28	287
Total	**155**	**149**	**82**	**76**	**154**	**9**	**116**	**91**	**832**

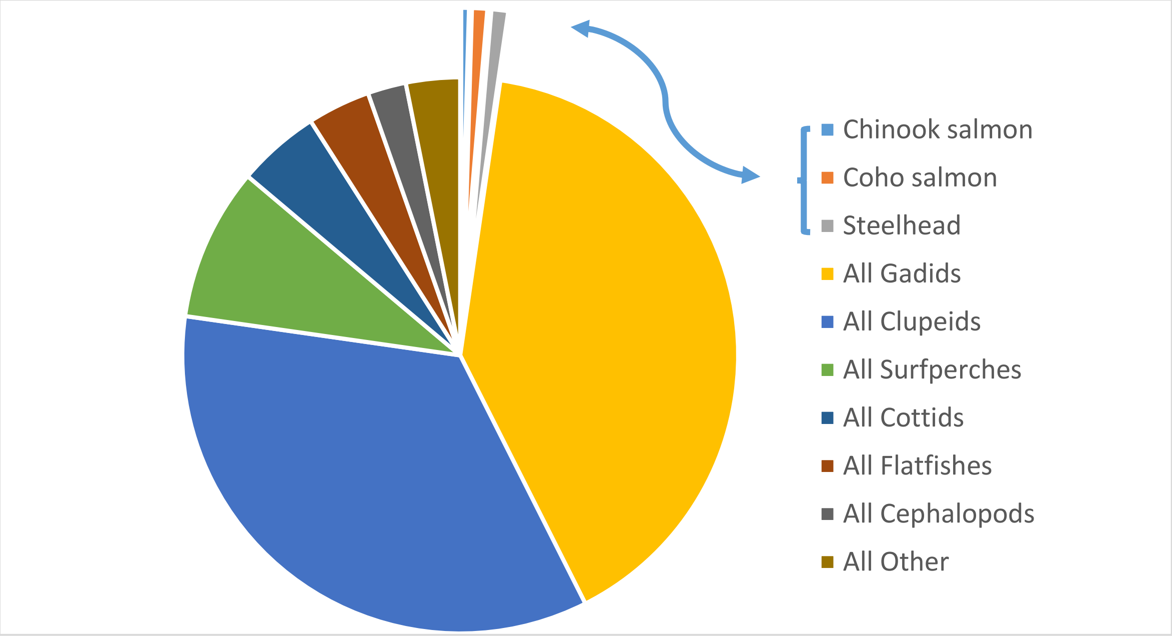
[1] Scat from seal (B1946) captured at Orchard Rocks

[2] Only scat collected at Colvos Rocks during eight capture attempts
[3] Only scat collected at Orchard Rocks during seven capture attempts

These sample sizes may seem adequate for determining prey composition, but it is important to keep in mind that we are only sampling a small percent of the available scats and animals in the region. One way to emphasize this point is to compare the number of scats collected by haulout and the number of animals associated with those haulouts. Although the number of animals at a given haulout is constantly in flux, if we use approximate estimates from aerial surveys, it is evident that we are sampling a very small proportion of the animals/scats. For example, there are approximately 180 animals associated with the Woodard Bay haulout yet, on average, we collect fewer than 10 samples from that site per visit (range = 0 – 17). In other words, the majority of scats are deposited in the water and not on land so are never available for collection. The outcome of this relatively small sample could be unrepresentative results of actual seal diet, especially for prey species that are only found in a small percent of the total diet. Given the relatively even distribution of samples in space and time, we have no reason to expect these results to be unrepresentative.

From these 832 harbor seal scat samples, we identified 77 different prey species using DNA. Overall, diet was dominated by fish, but also included an unknown crustacean, and three species of cephalopods (Pacific red octopus, giant Pacific octopus, California market squid). Ten fish species composed over 76% of the total diet based on our DNA results and included Pacific hake, Plainfin midshipman, Pacific herring, Pacific staghorn sculpin, shiner surfperch, Northern anchovy, buffalo sculpin, Pacific tomcod, starry flounder and American shad. We detected DNA of five salmonid species in harbor seal feces including Chinook, chum, coho, cutthroat trout, and steelhead.

Figure 3. Percent diet by species or species group (e.g., Gadids) in May and June combined (n = 149) using DNA, emphasizing the salmonid portion of the total diet.



During outmigration from April to June, steelhead DNA was identified in 3 scats during a seven day window in early May. In other words, steelhead DNA was identified in three of 149 samples (2.0% occurrence) and comprised 0.98% of the population diet for that month. Of the total DNA sequenced within these three scats, the percent steelhead was 2.5%, 43% and 100%. It is important to not emphasize the results from a single sample. For example, it would not be appropriate to state that the sample with 100% steelhead DNA is an example of diet specialization by that seal. When we compare the hard part and DNA results from these three samples containing steelhead DNA, we see very different results between the two methods. Note that sample Pv16-252 with 100% steelhead DNA also had hard parts from walleye pollock, Pacific tomcod, sculpin and Clupeid that were either not detected genetically or the DNA results from these fish were discarded because they represented < 1% of the total number of prey DNA sequences for that sample. Small DNA percentages are discarded following the standard Bioinformatic protocol for scat samples. This step in the protocol is included to reduce the potential impacts of low copy-number DNA contaminates from biasing DNA metabarcoding results. Steelhead DNA was also detected in seal feces outside the juvenile outmigration window in February (0.33%), July (0.13%), and August (0.66%).

Table 3. Comparison of DNA and hard part results for the three samples with steelhead DNA.

Sample Identification	DNA Results	Hard Part Results
Pv16-252	Steelhead (100%)	Oncorhynchus sp., pollock, tomcod, staghorn sculpin, and clupeid spp.
Pv16-462	Steelhead (43%), Chinook, coho, herring, and butterfish	Chinook otolith, Juvenile Onorhynchus sp., herring, and Pacific pomano
Pv16-471	Steelhead (2.5%), shiner perch, staghorn sculpin, herring	cephalopods, shiner perch, midshipman, herring, and gadid species

DNA analyses found Chinook salmon occurred in all months (range = 0.45-2.11% of population diet), Coho salmon ranged from 0.13 - 1.19% in May – August (but also occurred in January), chum salmon was present in most months and primarily ranged from 0.01 - 1.83% of population diet. However, in January it represented 11.7% of the total population diet. See Figures 1-7 that follow.

Caveats:

- These results are preliminary and may change as we perform additional data checks
- Data should not be presented for smaller windows of time than a month (e.g., 2 weeks) because of sample size issues discussed above.
- It is important to recognize that both DNA and hard parts methods are imprecise for different reasons and focusing on individual samples or small sample sizes is inappropriate especially for salmonids which we found are a minor component of seal diet.
- Reliance on a single dietary method (DNA or hardparts) is likely less ideal than using a combined approach. We found that DNA results appear to underrepresent certain dietary items such as cephalopods. Instead, we recommend working on a statistical approach that integrates the

results from both methods. Given the apparent strong and generally consistent relationships between DNA and hard parts results, we are hopeful that such an approach would work.

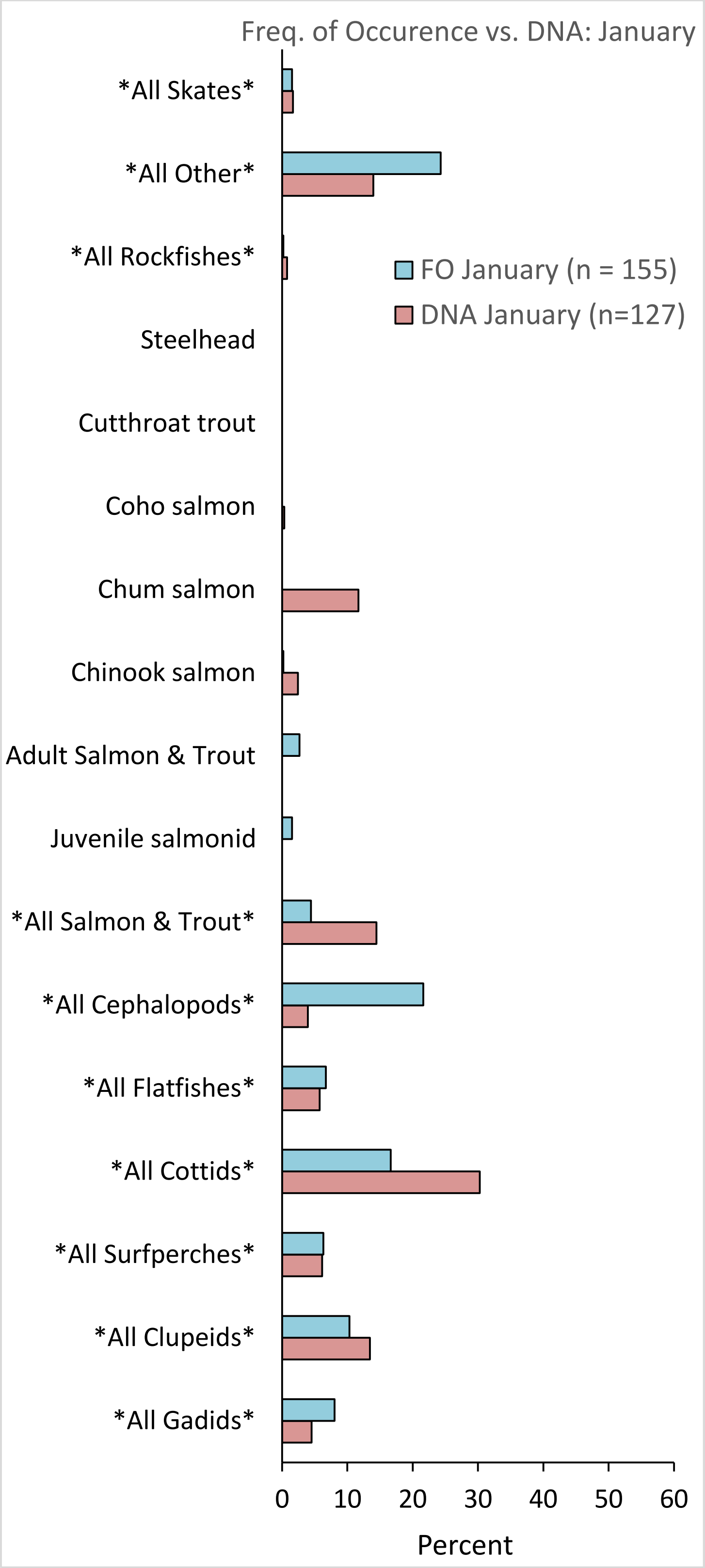
- This report represents samples from only one year and do not represent the inter-annual variation in diet that previous studies have shown.
- Finally, all of these estimates have variances, which will be presented in the final report/manuscript. It is critical to include this uncertainty in all population and ecosystem modelling to accurately represent seal diet and the importance of outmigrating steelhead.

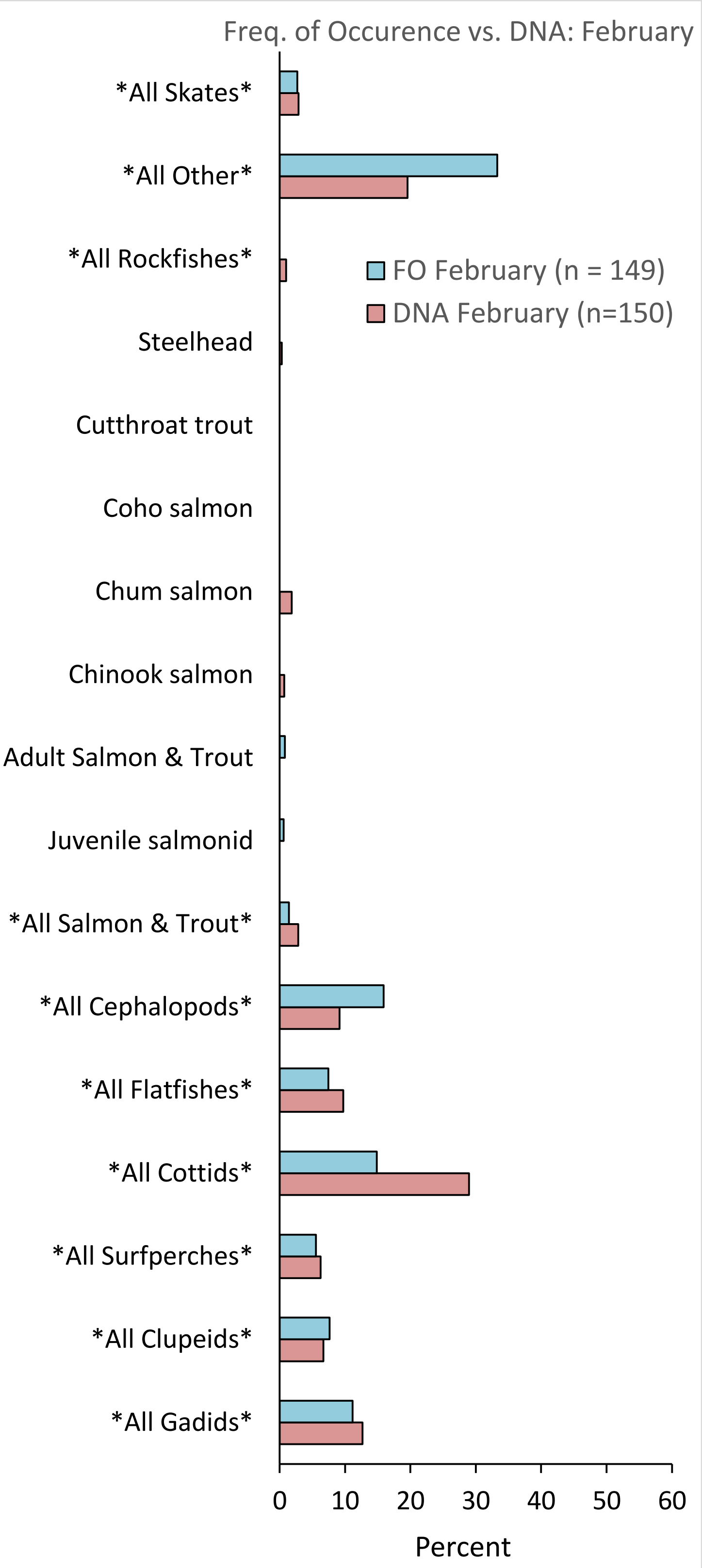
Acknowledgements

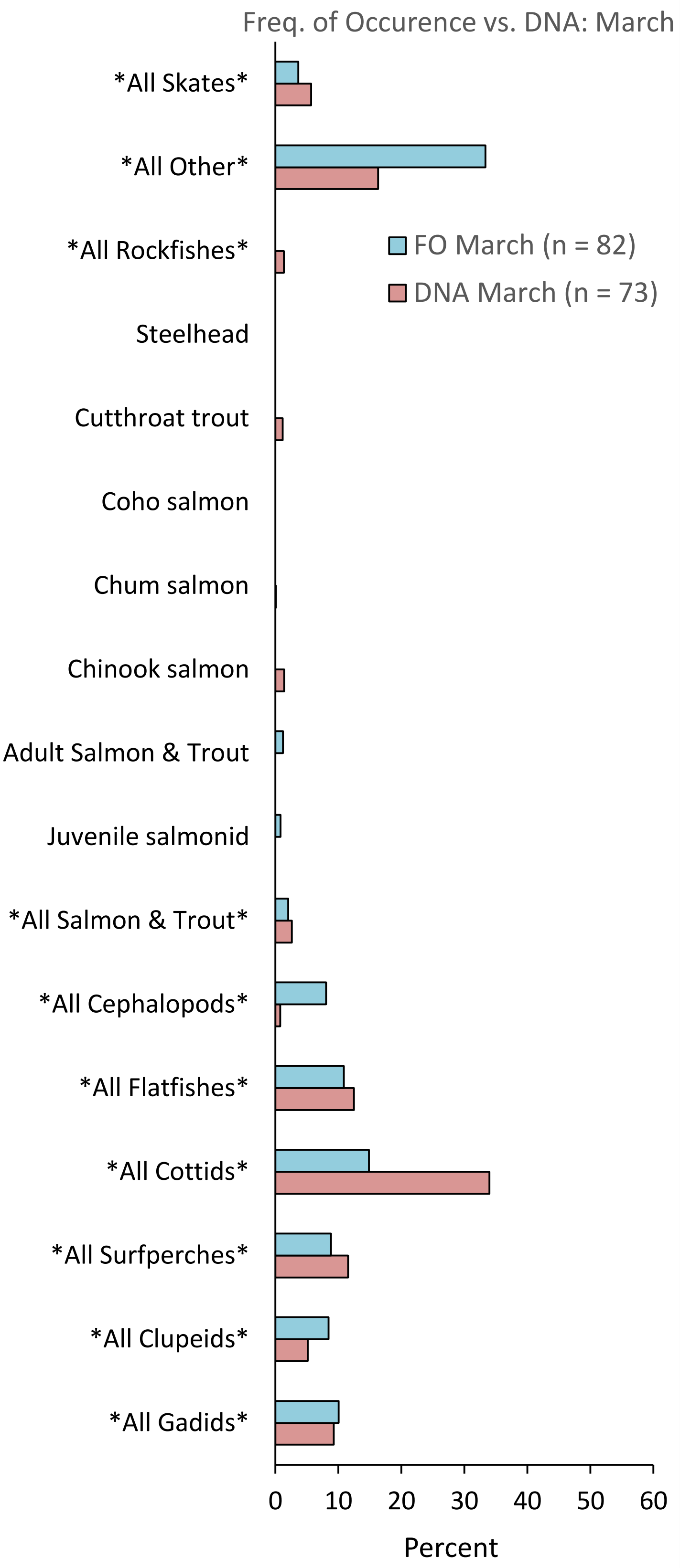
We thank WDFW Genetics Lab; Josh Oliver and Dyanna Lambourn with WDFW; and Chris Ellings and Jed Moore with Nisqually Fisheries.

This study is part of the Salish Sea Marine Survival Project: an international, collaborative research effort designed to determine the primary factors affecting the survival of juvenile chinook, coho and steelhead survival in the combined marine waters of Puget Sound and Strait of Georgia (marinesurvivalproject.com). The Puget Sound steelhead marine survival research component is overseen by the Washington Department of Fish and Wildlife (WDFW); the studies were developed and implemented through a workgroup including WDFW, NOAA Fisheries, US Geological Survey, the Nisqually Tribe and private contractors; and the effort was coordinated by nonprofit, Long Live the Kings. Funding was provided by Washington State with equal in-kind contributions by those participating in the research.

Figures 1-7. Percent DNA and Frequency of occurrence by species or species group.



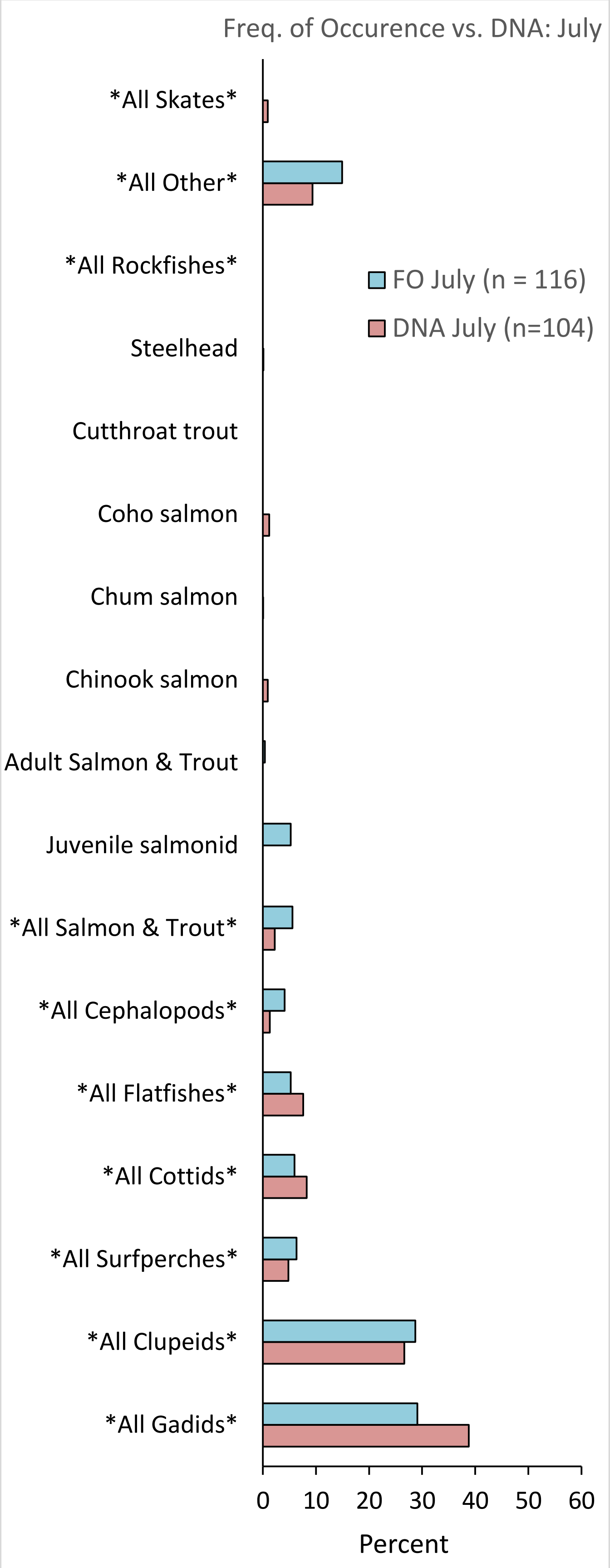


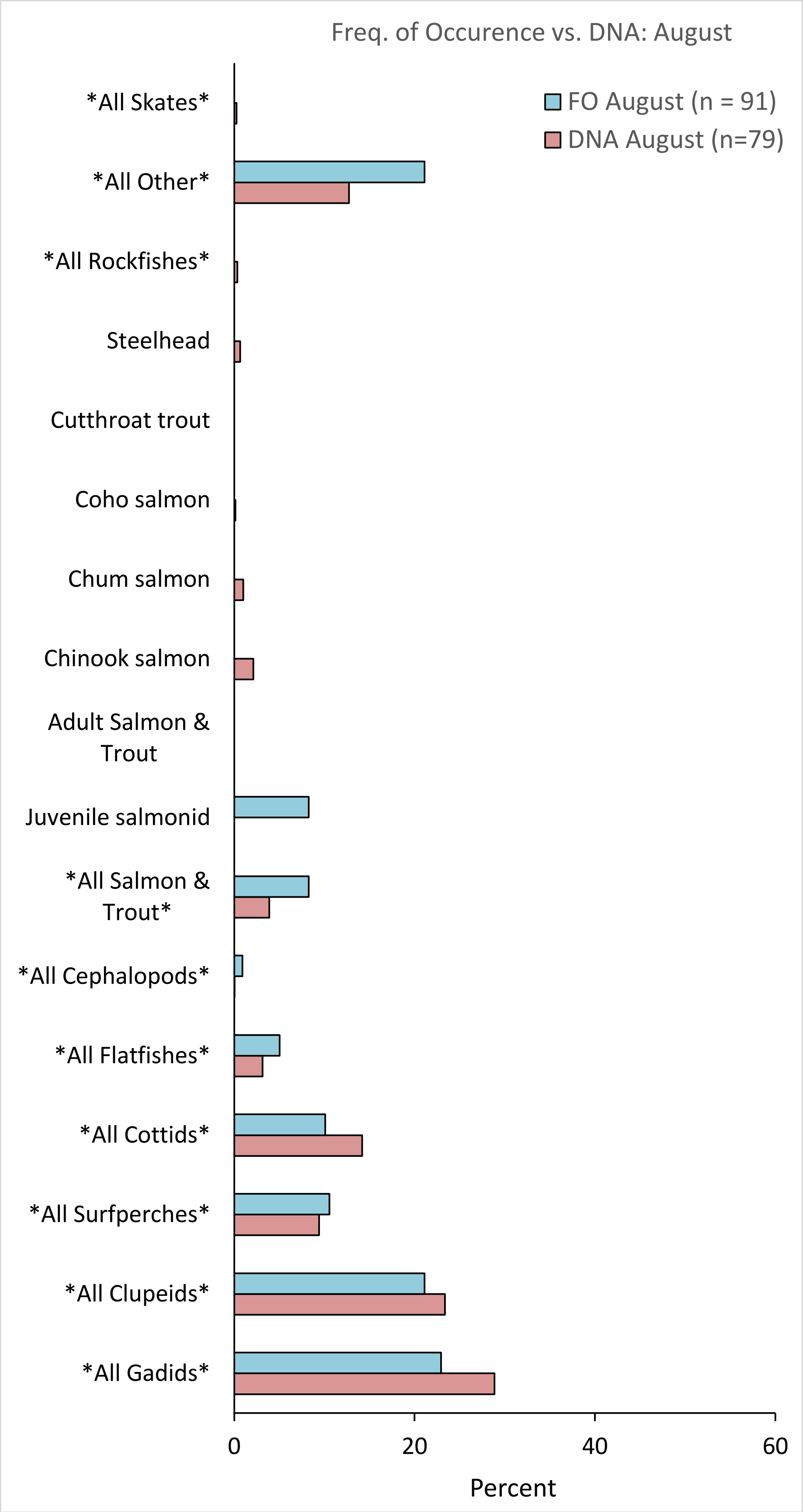


Freq. of Occurence vs. DNA: March

Freq. of Occurence vs. DNA: April


Freq. of Occurence vs. DNA: May & June
All Skates
All Other
All Rockfishes
Steelhead
Cutthroat trout
Coho salmon
Chum salmon
Chinook salmon
Adult Salmon & Trout
Juvenile salmonid
All Salmon & Trout
All Cephalopods
All Flatfishes
All Cottids
All Surfperches
All Clupeids
All Gadids
FO May+June (n = 163)
DNA May+June (n = 149)
0
10
20
30
40
50
60
Percent


Freq. of Occurence vs. DNA: July
All Skates
All Other
All Rockfishes
Steelhead
Cutthroat trout
Coho salmon
Chum salmon
Chinook salmon
Adult Salmon & Trout
Juvenile salmonid
All Salmon & Trout
All Cephalopods
All Flatfishes
All Cottids
All Surfperches
All Clupeids
All Gadids
FO July (n = 116)
DNA July (n=104)
0
10
20
30
40
50
60
Percent


Freq. of Occurence vs. DNA: August
FO August (n = 91)
DNA August (n=79)
All Skates
All Other
All Rockfishes
Steelhead
Cutthroat trout
Coho salmon
Chum salmon
Chinook salmon
Adult Salmon & Trout
Juvenile salmonid
All Salmon & Trout
All Cephalopods
All Flatfishes
All Cottids
All Surfperches
All Clupeids
All Gadids
0
20
40
60
Percent

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Study 7: Fish characteristics and environmental variables related to marine survival of Western Washington State steelhead trout (*Oncorhynchus mykiss*)

Kathryn Sobocinski[2,3], Neala W. Kendall[1] and Correigh M. Greene[2]

[1]Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501-1091 USA

[2]Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration Fisheries, 2725 Montlake Boulevard E., Seattle, WA 98112-2907

[3]Long Live the Kings, 1326 5th Ave. Ste. 450, Seattle, WA 98101

Introduction

Marine survival among Salish Sea Chinook and coho salmon and steelhead has declined in recent decades. While the cause is likely multi-faceted, increased pinniped abundance, changing foodweb dynamics, and anthropogenic impacts to inland marine waters have all been hypothesized as contributing factors. To better understand the decline in steelhead marine survival, we conducted a retrospective analysis aimed at evaluating how changing conditions in the Salish Sea and the Pacific Ocean over the last four decades have related to rates of steelhead marine survival.

Methods

We gathered data necessary to estimate smolt-to-adult return (SAR) rates, representing marine survival, for 12 (2 wild and 10 hatchery) Puget Sound populations from ocean entry years 1977 to 2014. We then developed hypotheses about steelhead marine survival based upon our mechanistic understanding of steelhead passage from their spawning streams to the Pacific Ocean and back to their natal systems to spawn (Table 1).

From these hypotheses we developed a suite of potential indicators from available time series (Table 2). Some of the hypotheses (those in italics) were unable to be assessed given a lack of applicable data. We selected indicators that are tied to the marine survival hypotheses, reflect changes to the environment of steelhead and are themselves changing over time, and that are available or could be derived for the period of time of interest (1970s-present).

We summarized potential indicator data, developed indicator time series, and assessed each time series for usefulness. Where appropriate, seasonal splits, lags, and other time-related adjustments were evaluated to find the most representative and useful form of the indicator. We assessed all indicators for collinearity and looked at correlations between our response variable, steelhead SAR time series, and each indicator. From this initial vetting, we selected a subset of indicators for use in statistical models.

Table 1. Hypotheses and sub-hypotheses related to steelhead marine survival. Those in italics could not be fully assessed due to lack of data availability.

HYPOTHESES	**SUB-HYPOTHESES**
H1: PREDATION	H1a: An increase in harbor seals in Puget Sound has led to increased predation of steelhead
	H1b: Orca presence in Puget Sound reduces predation of steelhead by harbor seals
	H1c: Increased predation by seabirds, other mammal predators, and piscivorous fishes on steelhead
H2: FORAGE FISH	H2a: Forage fish abundances serve as predation buffers for steelhead
	H2b: High abundances of young-of-year forage fish (especially anchovy) are beneficial as prey for predators who would otherwise target steelhead
H3: PRIMARY PRODUCTION	*H3a: Shift in primary production from diatoms to dinoflagellates has reduced primary production with subsequent cascades through the food web, increasing predation on steelhead*
H4: ESTUARY/EARLY OCEAN CONDITION: RIVER FLOW AND OCEANOGRAPHY	H4a: Ocean conditions, reflected by the Pacific Decadal Oscillation and North Pacific Gyre Oscillation, have influenced survival of steelhead
	H4b: High river flows result in poor steelhead survival due to adverse hydrological conditions
	H4c: High Puget Sound turbidity (from river flow or primary production) increases steelhead survival by masking them from predators
H5: HATCHERY SALMONID RELEASES	H5a: Density-dependent factors such as attracting predators, predator swamping, or competition affecting steelhead survival

Table 2. Potential indicators related to the hypotheses described in Table 1. Those in italics were evaluated but not pursued further due to data quality issues or lack of contrast in the dataset.

HYPOTHESES	INDICATORS
H1: PREDATION	Abundance of harbor seals (Jeffries et al. 2013, WDFW)
	Resident orca abundance (Chasco et al. 2017)
	Fish abundance included Hake, Walleye Pollock, Spiny Dogfish, English Sole, and Spotted Ratfish, 1987-2007 (WDFW)
H2: FORAGE FISH	Herring spawning stock biomass (WDFW)
H3: PRIMARY PRODUCTION	*Satellite derived chlorophyll a for recent years (Brandon Sackmann, unpublished data)*
H4: ESTUARY/EARLY OCEAN CONDITION: RIVER FLOW AND OCEANOGRAPHY	Puget Sound Water Quality: SST (Sea Surface Temperature), *salinity, dissolved oxygen, pH, chlorophyll a, light transmissivity* (Washington State Department of Ecology)
	Oceanographic indicators: SST, salinity, NPGO, PDO, MEI, PNI, NPI, upwelling index, date of spring transition
	River conditions: maximum flow from major rivers, spring flow, date of maximum flow, date of 50% cumulative flow, date of 75% of cumulative flow, duration of time between 25% and 75% of cumulative flow (USGS)
	Human population as an indicator of disturbance within the Salish Sea (US Census)
H5: HATCHERY SALMONID RELEASES	Total abundance of hatchery salmonid released in an outmigration year (WDFW)
	Total abundance of hatchery subyearling Chinook released (the largest component of hatchery production) in a year (WDFW)
	Average release date of hatchery subyearling Chinook (WDFW)
	Total number of hatchery yearling Chinook releases (WDFW)
	Average release date of hatchery yearling Chinook (WDFW)
	CV of hatchery subyearling Chinook release date (WDFW)
	Pink salmon abundance/presence absence (WDFW)

To relate SAR rates to the indicators we used generalized additive models (GAMs), which use smooth functions of the indicators to model the SARs. The model form utilized the binomial distribution for the response (SAR=Runsize/Smolt Outmigrants). All model fitting was done using un-biased risk estimator (UBRE) and validated with REML (restricted maximum likelihood). We constrained the model to avoid over-parameterization, given the limited SAR time series and the large number of potential explanatory variables. All variables were Z-scored (the observed value minus the mean from the time series, divided by the standard deviation of the time series) prior to model fitting. Using model selection, we evaluated indicators individually and in combination, thereby addressing possible interactions and cumulative effects.

Results

We evaluated multiple GAMs with a suite of indicators including: pinniped (H1, Table 2) and herring abundances (H2), oceanographic processes, marine water conditions including freshwater inflow (H4), and hatchery salmonid releases (H5). Model selection was based upon AIC$_c$. The best-fitting model was:

> SAR (Runsize/Smolts) ~ Year + *s*(Seal Abundance) + *s*(SST in Puget Sound) +
> *s*(NPI) + *s*(PDO) + *s*(Abundance of Hatchery Subyearling Chinook Releases)
> + *s*(CV of Hatchery Subyearling Chinook Release Date)

where *s* represents a smoothed term. The model fit is shown in Figure 1.

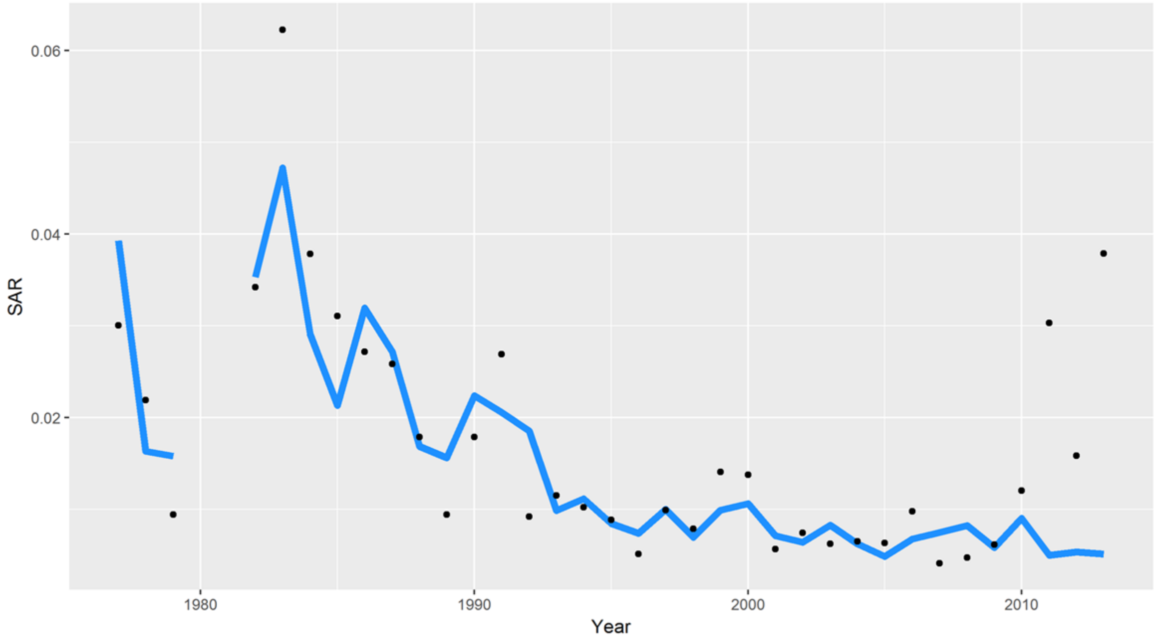

Figure 3. Model output (blue line) plotted with SAR observations for all Puget Sound steelhead populations. 1980 and 1981 are missing SAR data, so those years were omitted from the analysis.

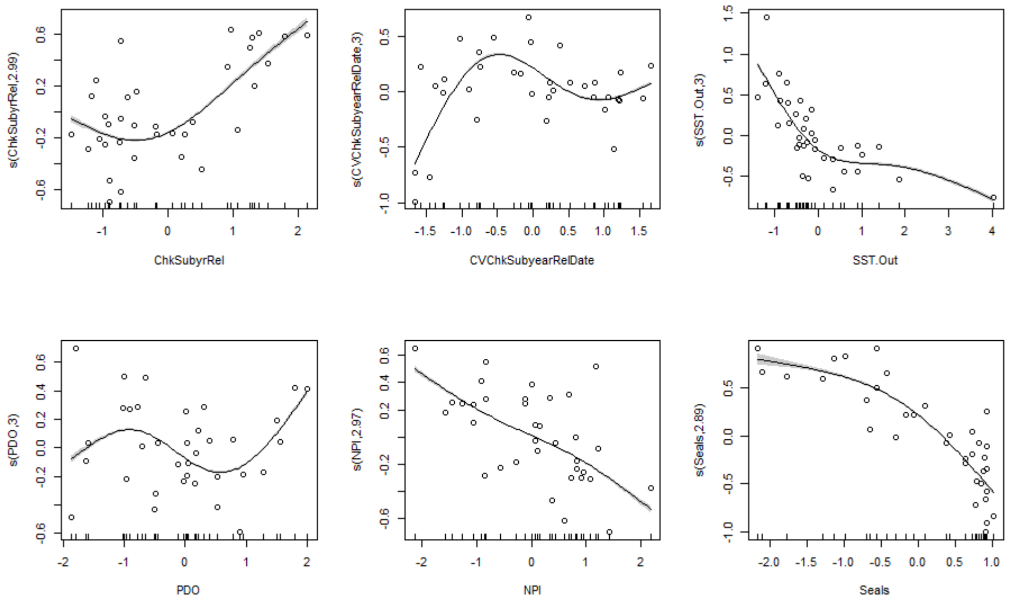

Figure 4. GAM partial correlation plots showing the effect of each indicator (x-axis) on SAR (y-axis).

Conclusions

The results of these models point to multiple possible drivers of the declines in steelhead marine survival rates since the late-1970s (Figure 2). Seal abundance was strongly negatively related to SAR, while Puget Sound SST and NPI values also had negative effects. PDO and the CV of hatchery subyearling Chinook release date had more moderate relationships with SAR, with variable influence. Finally, there was a positive correlation between abundance of outmigrating hatchery Chinook subyearlings and steelhead SAR. Neither herring spawning biomass nor aspects of river flow added explanatory power to the model and were not included in the best fitting model. There were not enough data to assess the explanatory power of Puget Sound turbidity.

We will continue to refine the models, including considering temporal autocorrelation in the model structure and further assessing the strength of the covariates in driving the model output. We will also gather additional data as they come available, including extending the SAR and indicator time series and including any new indicator time series.

Study 8: Population, habitat, and marine location effects on early marine survival and behavior of Puget Sound steelhead smolts

Megan E. Moore and Barry A. Berejikian

Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration Fisheries, P.O. Box 130, Manchester, 98353, USA

Steelhead trout (Onchorhynchus mykiss) smolts suffer high mortality rates during their rapid migration through the Salish Sea. Among-population variability in mortality rates may reflect (1) genetic fitness variation among populations, (2) freshwater environmental effects on fish condition, or (3) differences in local marine conditions upon seawater entry. A reciprocal transplant experiment was conducted to separate the influence of freshwater effects (combined effects of population and freshwater environment) from effects of local marine conditions on survival of two Puget Sound steelhead populations. Steelhead smolts from the Green River in Central Puget Sound (urbanized and hatchery-influenced) and the Nisqually River in South Puget Sound (less urbanized; no hatchery influence) were tagged with acoustic telemetry transmitters and released back into their natal river or transported and released into the other river. Population of origin had little influence on probability of surviving the migration through Puget Sound. However, smolts released into the Green River had higher survival through Puget Sound (17%) than smolts released into the Nisqually River (6%); the extra 64-km migration segment for the Nisqually-released fish accounted for most of the difference between the two release locations. Neither fork length nor translocation influenced survival, though release date did affect survival of Nisqually population smolts regardless of their release location. Residence time and behavior in the two estuaries were similar, and no effects of population of origin or release date were evident. Marine travel rates also did not differ between populations, release dates, or release locations. This study indicates that mortality occurring in the Salish Sea is likely driven by processes in inland marine environments, more so than intrinsic effects of population or freshwater-rearing environments.

(Publication: Moore, M. E., and B. A. Berejikian. 2017. Population, habitat, and marine location effects on early marine survival and behavior of Puget Sound steelhead smolts. Ecosphere 8(5):e01834. 10.1002/ecs2.1834)

Study 9: Steelhead smolt releases from Skagit River used to estimate detection efficiency of Strait of Juan de Fuca acoustic telemetry line

E.J. Connor[1], E. Jeanes[2], and C. Morello[2]

[1]Environmental Affairs Division, Seattle City Light, 700 Fifth Avenue, Seattle, WA 98124-4023

[2]R2 Resource Consultants, Inc., 15250 NE 95th Street Redmond, WA 98052

An experimental release of acoustically tagged steelhead smolts from the Skagit River was completed in the spring of 2014 to estimate the detection efficiency of the Strait of Juan de Fuca (SJDF) acoustic receiver line. This study was also conducted to identify the migratory routes of Skagit steelhead smolts through the Puget Sound, to compare smolt survival rates of among these routes. We surgically implanted acoustic transmitters (tags) in 100 steelhead smolts obtained at the WDFW Marblemount Hatchery in the upper Skagit River watershed. We tagged 50 of the smolts using Vemco V7-2L tags, which was the same type of tag used for tracking the marine migration survival of steelhead smolts released from the Green and Nisqually rivers in 2014. The remaining 50 smolts were tagged with larger and higher power Vemco V9-2H tags, which we determined from prior studies as having a 100% detection efficiency at the SJDF line. The SJDF line is the most important receiver array in the Puget Sound, since it is the largest and the last array that can detect tagged fish prior to entering the Pacific Ocean.

The tagged steelhead smolts were released into the Skagit from the Marblemount Hatchery on May 13, 2014. These fish migrated 127 km down the Skagit River from the release site to the Skagit Bay estuary in an average of 12 days. A total of 33 of the 100 tagged smolts were detected in Skagit Bay, indicating that freshwater survival rate over this distance was approximately 33%. We determined that the detection efficiency of the two receiver arrays deployed in north end and south end of Skagit Bay was close to 100% based upon a comparison of V7 and V9 detection rates. A total of 10 tagged Skagit steelhead smolts were detected at the SJDF line prior to their outmigration into the Pacific Ocean. Of these tags, four were V7-2L tags and six were V9-2H tags. Given that the detection efficiency of the V9-2H tags was 100%, the estimated detection efficiency of V7-2L tags at the SJDF line was 66.7%. The average travel time for the Skagit steelheads smolts from Skagit Bay to the SJDF line was five days, with the travel distance for these smolts averaging 163 km over two possible migration routes through the Puget Sound. The average marine survival rate for steelhead smolts (Skagit Bay to SJDF line) was approximately 33%, while the average combined freshwater and marine survival rate from the Marblemount Hatchery to SJDF line was approximately 12%.

We also compared the marine survival rates of Skagit steelhead smolts between two possible migration routes: 1) north through Deception Pass and then west to the Strait of Juan de Fuca for a travel distance of 119 km; and 2) south through Saratoga Passage along the east side of Camano Island and then northwest through Admiralty Inlet to the Strait of Juan de Fuca for a travel distance of 207 km. A total of 18 of the 33 tagged smolts detected in Skagit Bay migrated via the northern route, while 15 of the 33 smolts migrated via the southern route. This means that 55% of the smolts migrated the shorter northern route via Deception Pass, while 45% of the smolts migrated the longer southern route via Saratoga Passage and Admiralty Inlet. The estimated marine survival rate for Skagit steelhead smolts migrating the northern route was 39%, while the estimated marine survival rate for smolts migrating the southern route was approximately 23%. This finding suggests that marine mortality increases as migration distances increase, which is consistent with predation as the major source of mortality to steelhead smolts in the Puget Sound. The overall estimated survival rate of Skagit steelhead smolts migrating through the Puget Sound in 2014 was 36%.

Study 10: *Nanophyetus salmincola* infection and toxic contaminant exposure in outmigrating Steelhead Trout from Puget Sound, Washington: implications for early marine survival

M. F. Chen[1], S. M. O'Neill[2], A. J. Carey[2], R. H. Conrad[1], B. A. Stewart[1], K. R. Snekvik[3], G. M Ylitalo[4] and P. K. Hershberger[5]

[1]Northwest Indian Fisheries Commission, 6370 Martin Way E., Olympia, WA 98670

[2]Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501

[3]College of Veterinary Medicine, Washington State University, Pullman, WA 99164

[4]Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Boulevard E., Seattle, WA 98112 USA

[5]U.S. Geologic Survey – Western Fisheries Research Center, Marrowstone Marine Field Station, 616 Marrowstone Point Rd., Nordland, WA 98358

Outmigrating steelhead trout *Oncorhynchus mykiss* from four Puget Sound rivers, and associated marine basins of Puget Sound in Washington State were examined for the parasite *Nanophyetus salmincola* in 2014 to determine whether recent trends in reduced marine survival are associated with the presence of this pathogen. A subset of steelhead trout from three of these river-marine basin combinations was analyzed for the presence of persistent organic pollutants (POPs) to assess whether exposure to these contaminants is a contributing factor to their reduced marine survival. The prevalence and parasite load of *N. salmincola* were significantly higher in fish from central and southern Puget Sound than fish from river systems in northern Puget Sound. The proportion of steelhead trout samples with concentrations of POPs higher than adverse effects thresholds (AETs) or concentrations known to cause adverse effects was also greater for fish from the central and southern regions of Puget Sound than the northern region. Polybrominated diphenyl ether concentrations associated with increased disease susceptibility were observed for 10% and 40% of the steelhead trout sampled from central and southern Puget Sound regions, respectively, but none of the fish sampled from the northern region. The AET for polychlorinated biphenyls was exceeded in Steelhead Trout collected from marine habitats: 25% of the samples in the marine basins in the central and southern regions of Puget Sound, and 17% of samples from northern Puget Sound region.[31] Both *N. salmincola* and POP levels suggest adverse health effects on outmigrating steelhead from one southern and one central Puget Sound River that have lower early marine survival than a river system in northern Puget Sound.

(Chen M.F., S.M. O'Neill, A. J. Carey, R. H. Conrad, B. A. Stewart, K. R. Snekvik, G.M. Ylitalo, and P.K. Hershberger. (in press). Nanophyetus salmincola infection and toxic contaminant exposure in outmigrating Steelhead Trout from Puget Sound, Washington: Implications for early marine survival. Journal of Aquatic Animal Health.)

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[31] In 2015, TBiOS repeated contaminant analyses on individual steelhead from in-river habitats (collected at the smolt trap) on the Nisqually River that confirmed the 2014 findings: approximately one third of the steelhead whole-body samples had PBDEs at known CBR concentrations for increased disease susceptibility in salmonids.

Study 11. Effects of *Nanophyetus* on the swimming performance and survival of steelhead smolts AND studies to understand and manage the *Nanophyetus* cercaria

P.K Hershberger[1]

[1]United States Geologic Survey – Western Fisheries Research Center, Marrowstone Marine Field Station, 616 Marrowstone Point Rd., Nordland, WA 98358

I. Effects of *Nanophyetus* on Steelhead Health and Survival

Recent field surveillances indicated that outmigrating steelhead smolts in several south Puget Sound watersheds are infected with the digenean trematode *Nanophyetus salmonicola* at high prevalence and intensity (Chen et al Accepted). The apparent severity of these infections, especially in the Nisqually and Green / Duwamish Rivers, lead to the hypothesis that *Nanophyetus* may play a role as a proximate and / or ultimate factor contributing to the early seawater mortality of smolts after entering Puget Sound. This hypothesis was tested during 2016 using controlled laboratory and field based studies that were intended to investigate possible effects of *Nanophyetus* infection on:

1. the survival of steelhead smolts during their outmigration through Puget Sound,
2. the ability of steelhead smolts to survive transition from freshwater to seawater,
3. the swimming performance of infected steelhead smolts.

Steelhead swimming performance and survival studies were designed around the hypothesis that early-stage *Nanophyetus* infections compromise the health and survival potential of exposed fish more than later stage established infections, which typically manifest as encysted metacercaria in fish tissues. Therefore, the exposure history of all experimental steelhead used in these studies was well-defined by initiating all experiments with specific pathogen-free (SPF) steelhead from the WDFW Icy Creek facility (a known *Nanophyetus*-free cohort) and performing all cercaria exposures under controlled conditions in the laboratory. SPF steelhead were transported to tanks supplied with single-pass freshwater at the USGS – Marrowstone Marine Field Station on February 22, 2016.

Two groups of steelhead smolts were established in the laboratory, infected (exposed to *Nanophyetus* cercaria) and control (unexposed). Fish in each group were maintained in separate 6 ft diameter tanks (N = 232 smolts / tank) that were supplied with single pass freshwater. All fish were fed to satiation daily with BioOregon (Bio Olympic) pellet. Exposures of SPF steelhead smolts to *Nanophyetus* cercaria occurred daily over seven consecutive days from May 16 – 22, 2016 by shutting off the freshwater supply, lowering the water level to 1,000 L, and adding *Nanophyetus* cercaria to the appropriate tank; freshwater supply was resumed 4-8 hr after the daily addition of cercaria. Fish were handled similarly in the control tank, but cercaria were not added. *Nanophyetus* cercaria for these exposures were harvested from *Juga* sp. snails that were collected from the East Fork of the Satsop River, adjacent to the WDFW Bingham Creek Hatchery. Snails were stimulated to shed cercria in the laboratory by exposure to intense light. Over the 7 d exposure period, fish in the treatment group were exposed to approximately 1.1 million waterborne cercaria, with different exposure levels occurring each day (Table 1). These two groups of steelhead were then used as experimental animals to address the following three objectives.

Table 1. Daily profile of cercaria exposures for all infected smolts used in these studies

Date (2016)	Cercaria Exposure Day #	Estimated Cercaria Exposure Levels (Counts)
May 16	0	4,500
May 17	1	20,590
May 18	2	456,170
May 19	3	130,650
May 20	4	109,000
May 21	5	323,000
May 22	6	46,600
	Total	**1,090,510**

Objective #1: To determine the effect of Nanophyetus infections on the survival of steelhead smolts during their outmigration through Puget Sound.

The successful passage of infected and uninfected steelhead through Puget Sound and the Strait of Juan de Fuca was assessed by inserting hydroacoustic tags (Vemco V-7) into smolts from each treatment group, then releasing the fish and recording tag detections at three stationary hydrophone arrays located along the outmigration corridor. Tags were surgically implanted into the body cavities of 150 fish from each treatment group (infected and control) on May 11-13; 3-5 days prior to the onset of the cercaria exposures (Table 1). Smolts were released on released on May 23, one day after the final cercaria exposure. Fish were transferred to 50% seawater and transported to the NOAA Manchester Laboratory, where they were loaded onto a vessel and slowly released into Central Puget Sound along a line extending from Blakley Rock to north Elliot Bay. The mean *Nanophyetus* load in the posterior kidneys of the infected fish (232 metacercaria, range = 30 – 865 metacercaria, n = 81) was estimated from all the fish (mortalities and survivors) that were euthanized in Objectives #2 and #3 (described below). Presumed false positives occurred in only 3/78 negative controls that were examined with *Nanophyetus* loads $\leq$2 metacercaria / posterior kidney.

Successful detection of tagged steelhead at each of the stationary hydrophone arrays was slightly lower among the infected fish than among uninfected cohorts (Figure 1); however comparisons were not significantly different at any of the arrays.

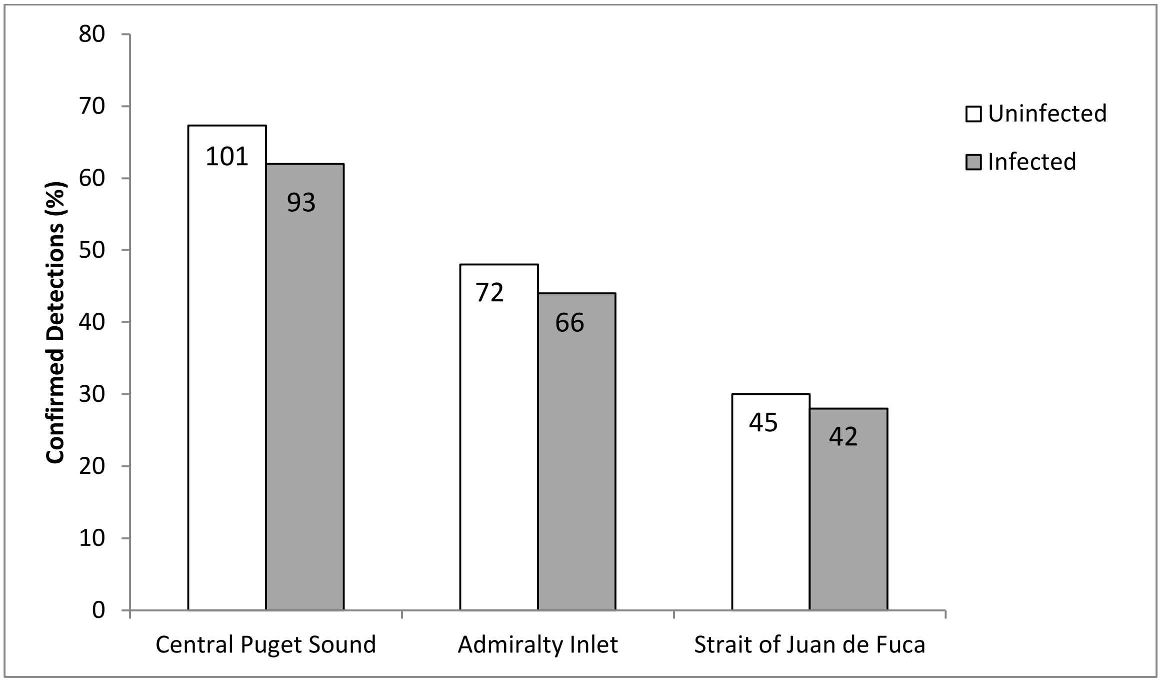


Figure 1. Detection of tagged steelhead through stationary hydrophone arrays located along the smolt outmigration route. N= 150 tagged steelhead were released in each group; numerals inside the bars indicate the number of successful detections at each array.

Objective #2: To determine the effect of Nanophyetus infections on the ability of steelhead smolts to survive transition from freshwater to seawater.

The ability of *Nanophyetus* infected smolts to survive seawater transition was assessed under controlled laboratory conditions. Infected and uninfected smolts were transferred to 760 L tanks that were supplied with single pass freshwater (N = 60 fish / treatment). Half the fish in each group (N=30) contained dummy Vemco V-7 tags and the other half (N=30) were untagged. Their gradual transition from freshwater to 50% seawater and 100% seawater occurred on May 23 and was coordinated with that of the smolts released for Objective #1. The mean *Nanophyetus* load in the posterior kidneys of the infected fish was 234 metacercaria (range = 55 – 865, N = 60); additionally metacercaria detections occurred in 3/60 negative controls, with extremely low loads of $\leq$2 metacercaria / posterior kidney. It is unknown whether these three low-load individuals represented false positives or were true low-intensity positives that may have resulted from exposure to cercaria at the Soos Creek Hatchery before the fry were moved to Icy Creek Ponds for rearing. Mortality was slightly higher among infected fish (6.7%) than among uninfected cohorts (0%); however, the differences were not significant (Figure 2). Among the infected fish, mortality was identical between the tagged and untagged groups (N = 2 mortalities each). *Nanophyetus* loads among the four mortalities ranged from 88-369 metacercaria / posterior kidney.

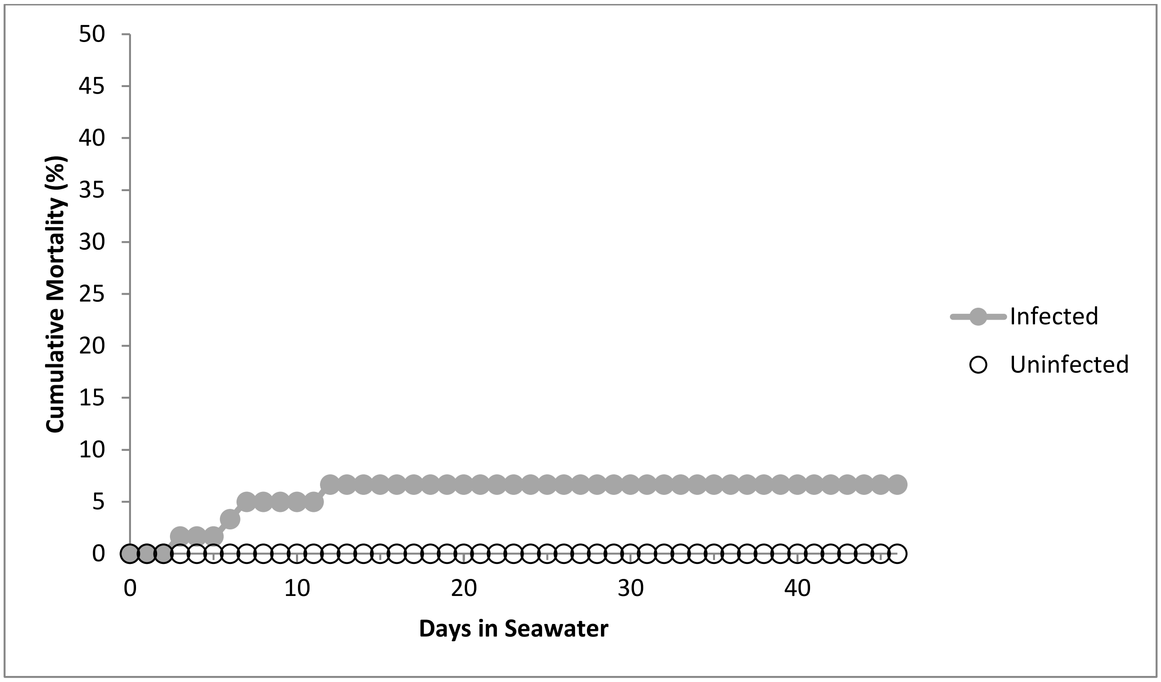


Figure 2. Cumulative mortality in 2 groups of steelhead smolts (*Nanophyetus* infected and uninfected) after their conversion to seawater. Each group contained 60 smolts.

Objective #3: To determine the effect of Nanophyetus infections on the swimming performance of infected steelhead smolts.

The impact of *Nanophyetus* infections on the swimming performance of infected steelhead smolts was tested by comparing the Critical Swimming velocity (U_{Crit}) between infected and uninfected groups in a Blazka respirometer. U_{Crit} is a measure of the maximum sustained swimming ability of a fish. Seawater transition occurred one day after the final *Nanophyetus* exposure, as in Objectives #1 and #2. To determine U_{Crit}, each fish was swum under a ramped protocol of increasing swimming velocities that included a 5 min. acclimation to the respirometer, followed by a 5 min. ramp to 3 body lengths (B.L.) / sec. Each fish then swam for 15 min periods at each velocity, ending in a 1 min ramp up to one addition B.L. /sec. This ramping protocol continued until the fish was exhausted; afterwhich the fish was transferred to a recovery tank. Each fish was swum twice, with the first swim occurring 1-20d after seawater entry and the second swim occurring 28-41d after seawater entry. The Critical Swimming Velocity for each fish was calculated as:

$$\mathbf{U_{Crit} = V_p + (T_f / T_i)\, V_i}$$

V_p= velocity at which the fish last swam for the full 15 min period
T_f = elapsed time to fatigue from the last velocity increase
T_i = time between velocity increases
V_i = Velocity increment (BL / sec)

The mean U_{Crit} was lower among *Nanophyetus*-infected smolts (5.15 B.L. / sec and 5.56 B.L. / sec) than among uninfected controls (5.48 B.L. / sec and 5.93 B.L. / sec) for both the first and second swims, respectively (Figure 3); however, the comparisons within each swimming period were not significantly different. Regressions between U_{Crit} and metacercaria load provided some indication of an inverse relationship between swim performance and metacercaria load (Figure 4), but it is recommended that these trends be further examined in additional studies using fish with higher metacercaria loads. Upon completion of the swimming trials, it was realized that the swimming performance of all fish (infected and uninfected) was relatively poor during the first several days after seawater entry. Therefore, several fish in the control group demonstrated low U_{Crit}'s during the first swim trial because they were among the first individuals that were swum following seawater transition. The U_{Crit} pattern among negative controls was much tighter during the second trial after all fish had opportunity to fully acclimate to seawater. Additionally, the fish with the highest metacercaria load in Swim #1 (768 metacercaria / kidney) died shortly after the first swim trial so a corresponding U_{Crit} does not exist for the second trial.

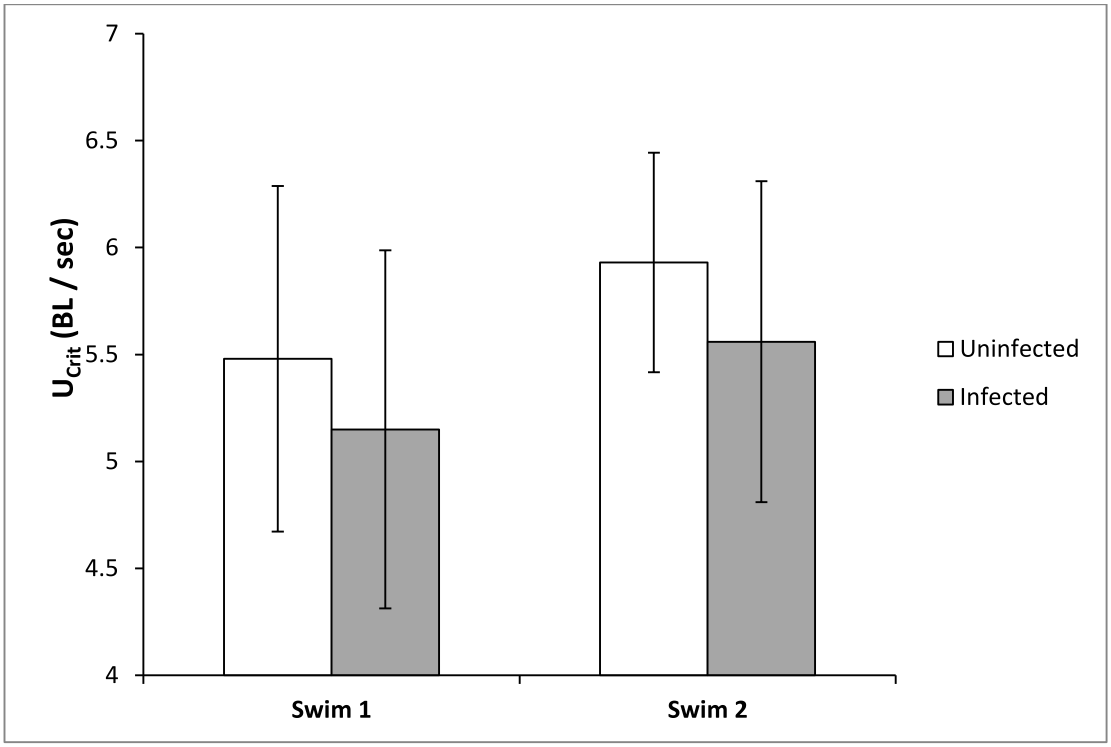
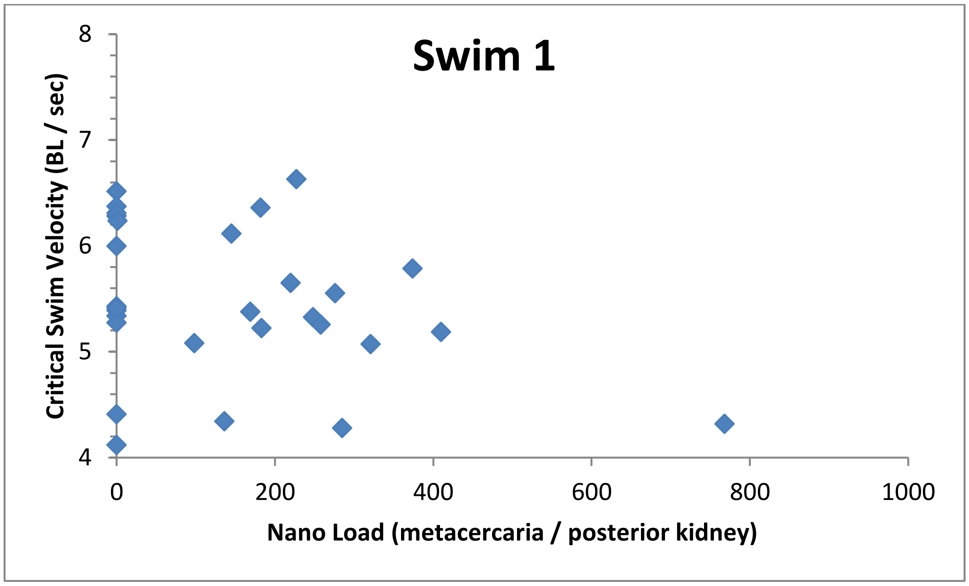


Figure 3. Comparison of mean U_{Crit} between infected and uninfected steelhead smolts in both swim trials. Swim #1 occurred 1-20 d after seawater entry and Swim #2 occurred 28-41d after seawater entry. N = 16 - 19 fish / treatment group. Error bars indicate 2 SD from the mean.



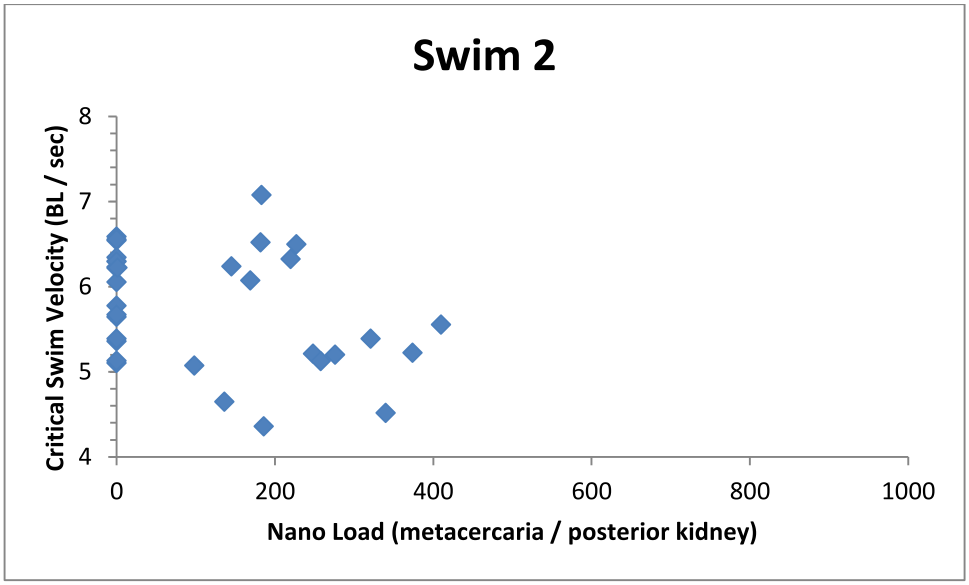


Figure 4. Influence of metacercaria load on U_{Crit}. The same fish were swum during the first and second trials.

II. *Nanophyetus* Cercaria Studies

Additional work was directed at understanding various aspects of *Nanophyetus* cercaria, the waterborne stage of the parasite that is responsible for initiating infections in a fish host. These cercaria studies were performed as a first step towards developing adaptive disease management strategies that may be focused on the prevention of salmonid exposure to the infectious stage of the parasite. Experiments were designed to develop and validate a quantitative polymerase chain reaction (qPCR) assay that is capable of:

1. Detecting *Nanophytetus* stages in water samples and

2. Testing the efficacy of therapeutics for their efficacy at killing waterborne cercaria.

Objective 4: Detection of Nanophyetus salmincola by quantitative PCR (Purcell et al. Accepted. Journal of Aquatic Animal Health)

We report the development and validation of two quantitative polymerase chain reaction (qPCR) assays to detect *N. salmincola* DNA in water samples and fish and snail tissues. Analytical and diagnostic validation demonstrated good sensitivity, specificity and repeatability of both qPCR assays. *N. salmincola* DNA copy number in kidney tissue was significantly correlated with metacercariae counts based on microscopy. Extraction methods were optimized for the sensitive qPCR detection of *N. salmincola* DNA in settled water samples. Artificially spiked samples suggested that the 1 cercaria / L threshold corresponded to an estimated $\log_{10}$ copies / L $\geq$ 6.0. Significant correlation of DNA copy number / L and microscopic counts indicated that the estimated qPCR copy number was a good predictor of the waterborne cercariae number (Figure 5). However, the detection of real-world samples below the estimated 1 cercaria / L threshold suggests that the assays may also detect other *N. salmincola* life stages, non-intact cercariae or free DNA that settles with the debris. In summary, the qPCR assays reported here are suitable for identifying and quantifying all life stages of *N. salmincola* that occur in fish tissues, snail tissues and water.

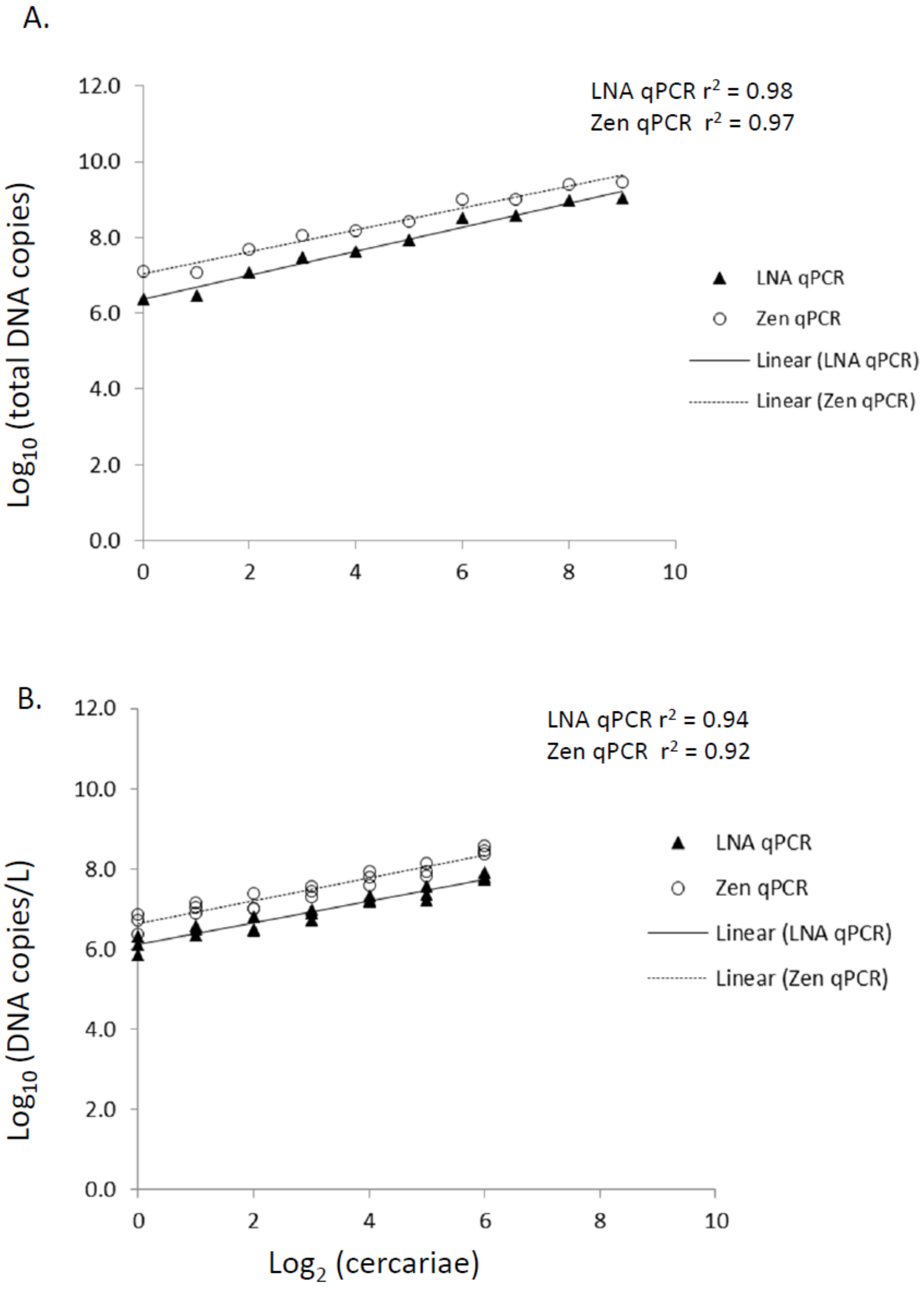


Figure 5. Relationship between number of cercariae and Nanophyetus salmincola DNA copy numbers determined by either the LNA or Zen quantitative PCR (qPCR) assays. (A) Direct extraction of a 2-fold number of cercariae ranging 1 – 512 (log2 0 – 9) cercariae. Only a single replicate was extracted at each cercariae number. (B) A 2-fold standard curve of cercariae ranging from 1 – 64 (log2 0 – 6), spiked into 1 L of creek water and extracted using the settling method. Each cercariae number was performed in triplicate.

Objective 5: Susceptibility of waterborne Nanophyetus salmonicola cercaria to chemotherapeutics (Hershberger et al. In Preparation)

In an effort to evaluate possible management strategies for minimizing steelhead exposure to *Nanophyetus*, we evaluated the efficacy of several possible therapeutants at killing waterborne cercaria. Exposures were performed in triplicate wells on a 24 well-plate by adding 10 newly-emerged cercaria to 1 mL of the appropriate dilution for each solution. Numbers of live cercaria were then evaluated at prescribed time intervals after exposure was initiated. Hydrogen peroxide (PEROX-AID®) was effective only at the highest concentration (100ppm), where only 17% of cercaria survived beyond the first hour (Figure 6). Exposure to Formalin resulted in 0% survival after 40 min at 83.5ppm and 30 min at 167ppm (Figure 7). Finally, only 20% of the cercaria survived for 10 min after immersion in brackish (15ppth) seawater and 0% survived for 10 min in full strength seawater (Figure 8). These results indicate that some chemotherapeutics may be efficacious for preventing *Nanophyetus* infections in hatchery situations, especially if administered during periods of elevated exposures to waterborne cercaria.

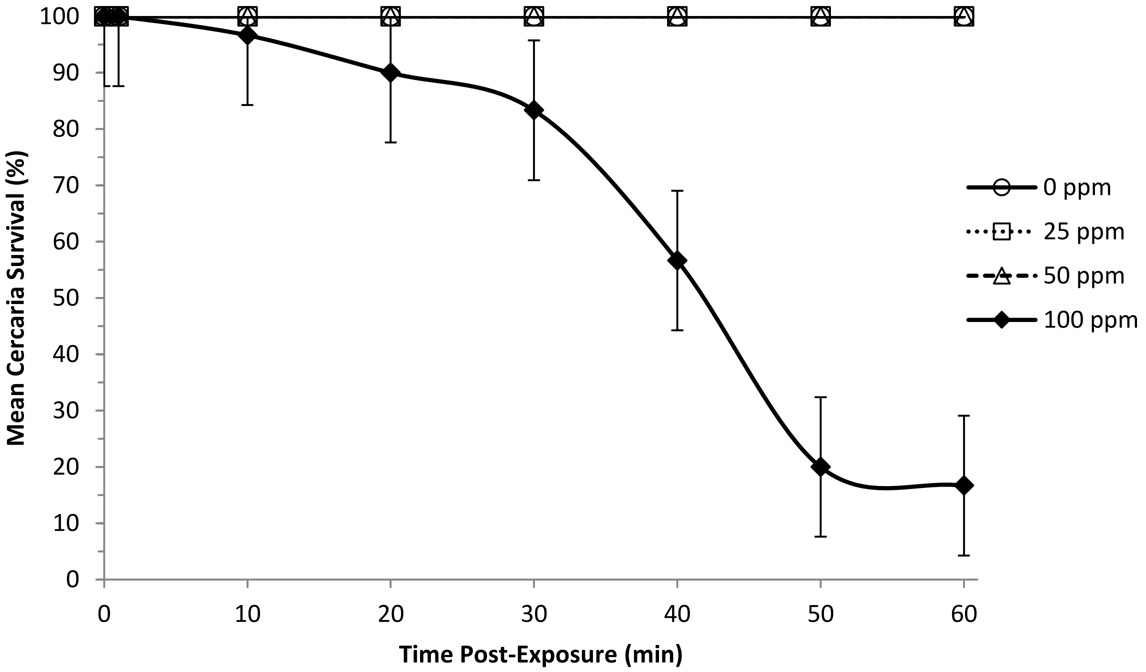


Figure 6. Survival of *N. salmincola* cercaria in dilutions of PEROX-AID®. Each data point represents the mean survival in triplicate wells, with each well containing 10 cercaria. Error bars indicate 2 SD from the mean.

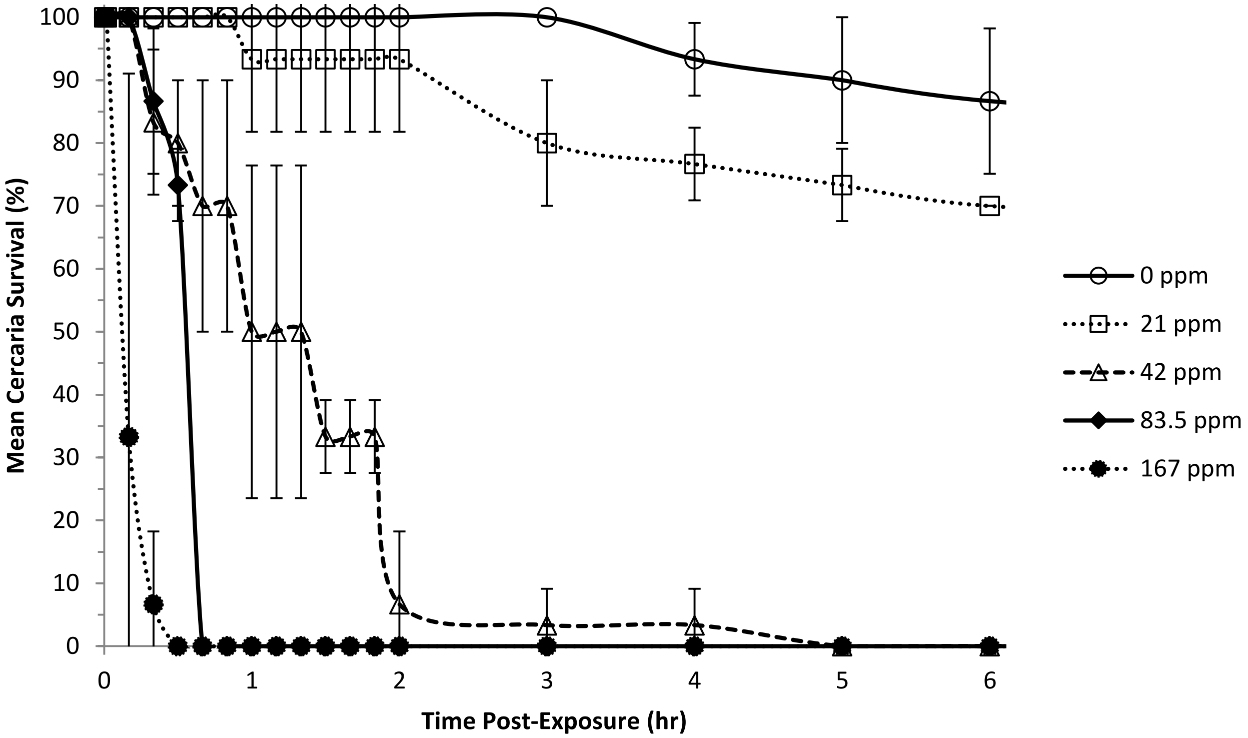


Figure 7. Survival of *N. salmincola* cercaria in dilutions of Formalin. Each data point represents the mean survival in triplicate wells, with each well containing 10 cercaria. Error bars indicate 2 SD from the mean. Mean survival after 24 hr was 53% (± 21%) at 0 ppm, 43.3% (± 38%) at 21ppm), and 0% in all other treatments.

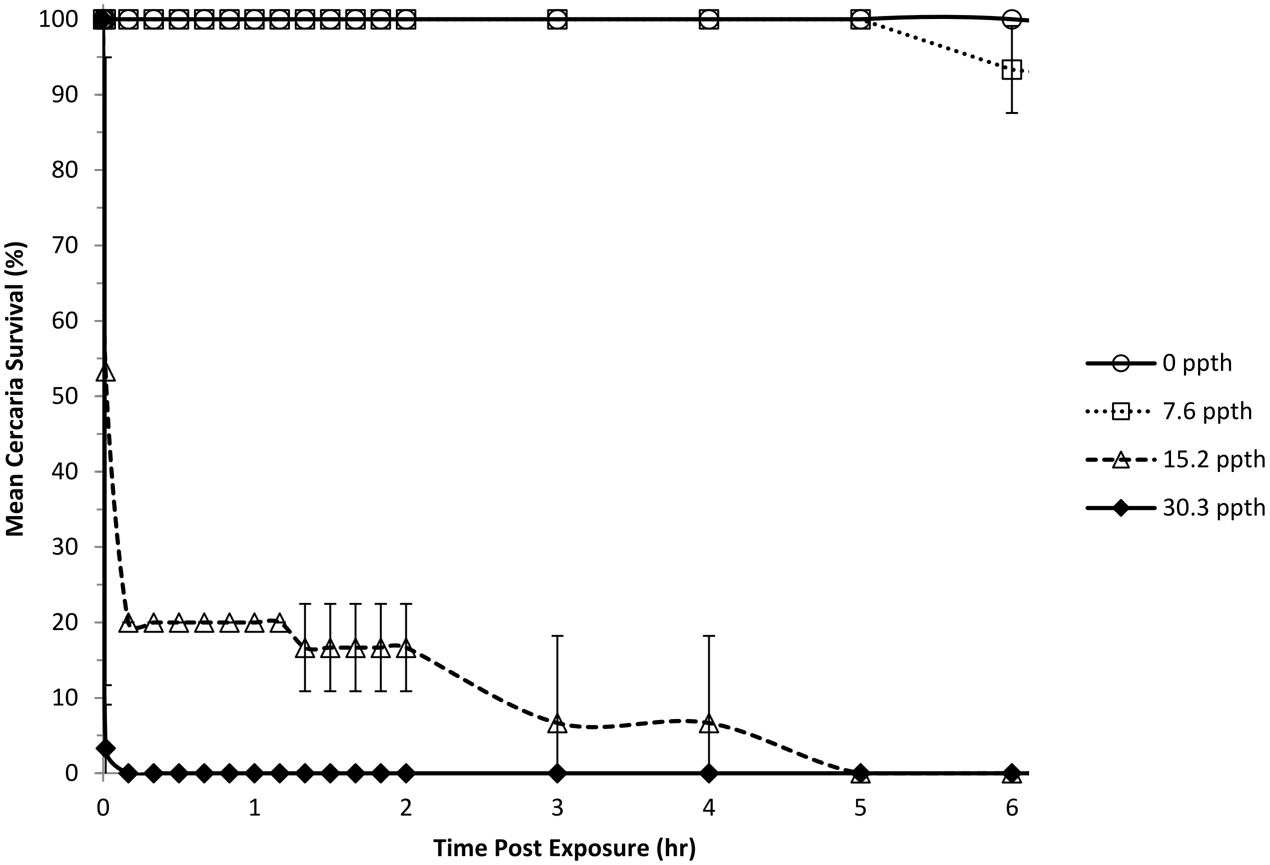


Figure 8. Survival of *N. salmincola* cercaria in dilutions of seawater. Each data point represents the mean survival in triplicate wells, with each well containing 10 cercaria. Error bars indicate 2 SD from the mean. Mean survival after 24 hr was 73% (± 5.8%) at 0 ppth, 53% (± 12%) at 7.6 ppth), and 0% in all other treatments.

Conclusions

Every measure of performance and survival we investigated was slightly lower among infected smolts compared to uninfected cohorts; however, none of the comparisons were statistically significant. This apparent lack of separation between groups may have occurred because of the relatively low parasite loads that were achieved during the controlled exposure studies. Because we wanted to examine the effects of relatively early *Nanophyetus* infections on smolt survival and performance, a limited window of time (7d) existed between parasite exposure and initiation of the various experiments. Although we exposed the fish to as many cercaria as we could during this period, the resulting metacercaria loads (mean ≈ 232 metacercaria / posterior kidney) were approximate 10 fold lower than those previously reported in outmigrating wild smolts from the Nisqually River (mean = 2,546 metacercaria / posterior kidney). If future cercaria exposure studies are performed, it is recommended that the exposures be performed differently to achieve comparable parasite loads to wild steelhead.

This study was designed largely around the hypothesis that steelhead smolts become exposed to a pulse of *Nanophyetus* cercaria during their final days prior to seawater entry, and this punctuation in new exposures results in compromised performance and survival. Since the completion of this study, we have completed quantitative daily assessments of waterborne *Nanophyetus* stages occurring in Soos Creek using a newly developed qPCR assay. The results from this new study indicate that the highest levels of cercaria exposure occur in the fall (September / October) and secondary exposures likely occur again in the spring, as steelhead smolts are outmigrating down the river. Therefore, it is recommended that future studies with *Nanophyetus* involve fish that experience the full seasonal spectrum of cercaria exposures.

Results from the *in vitro* chemotherapeutic studies indicate that effective prophylactic water treatments may be a viable option in some fish rearing facilities. Of these proposed treatments, low dilutions of Formalin may be the most viable option because FDA regulations may allow for protracted exposures during the natural cercaria shedding period. Additionally, it is worth noting that cercaria cannot tolerate relatively high salinities (> 15ppth), indicating that fish exposures to waterborne cercaria likely do not occur in the tidally-influenced reaches of the lower watersheds or estuaries

Study 12: Genome-wide association study of acoustically tagged steelhead smolts in the Salish Sea: measuring differences between survivors and non-survivors

Kenneth I. Warheit[1], Megan E. Moore[2], Barry A. Berejikian[2]

[1]Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501-1091 USA
email: kenneth.warheit@dfw.wa.gov

[2]Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration Fisheries, P.O. Box 130, Manchester, 98353, USA

Genome-wide association studies (GWAS; Balding 2006, Bush and Moore 2012) use genome scans to document relationships between phenotypes (e.g., survival) and genotypes (e.g., individual single nucleotide polymorphisms (SNPs), blocks of linked SNPs, or genes), based on population samples. GWAS are used in a wide variety of studies ranging from understanding diseases in humans (e.g., McCarthy et al. 2008, Hindorff et al. 2009), improving the agricultural production of domestic animals and plants (e.g., Daetwyler et al. 2009, Purdie et al. 2011), to documenting specific behavior or morphology in wild animals (e.g., Johnston et al. 2011). In salmonids, GWAS have been used, for example, to gain a better understanding of developmental rates and migratory behavior in steelhead/rainbow trout (e.g., Miller et al. 2012, Hecht et al. 2013, Johnston et al. 2014), and disease resistance in Atlantic salmon (e.g., Houston et al. 2012). In this study, we looked for associations between genomic signatures in steelhead smolts and their survival while out-migrating through Puget Sound, Washington State.

As part of their studies on the migratory behavior and survival of steelhead smolts in Puget Sound, from 2006 through 2010, and in 2014, Moore and Berejikian (e.g., Moore et al. 2010) surgically implanted steelhead smolts with acoustic transmitters. The acoustic signal from each transmitter is unique and enables the identification and general location of individual fish when their signal is detected by a receiver. The position of acoustic receivers varied from year to year, but for the purpose of this study, we defined the fate (Puget Sound mortality or survivor) of each smolt using the detection of their transmitter at receivers located at Hood Canal Bridge, Central Puget Sound, Tacoma Narrows, Admiralty Inlet, and Strait of Juan de Fuca (Figure 1). Specially, a fish was defined as a survivor if it was detected leaving Puget Sound at the Strait of Juan de Fuca receiver array. To be identified as mortality, first the fish needed to be detected leaving the river from which it was tagged (i.e., entering the marine waters of Puget Sound), and then being undetected at any of the aforementioned receivers[32]. During the surgical procedure to implant the transmitters, a small fin clip was taken from each smolt for DNA analysis. We selected from an initial list of 881 fin clips from Big Beef Creek, and Dewatto, Duckabush, Hamma Hamma, Skokomish, Green, and Nisqually rivers, 288 samples for DNA sequencing (Figure 1; Table 1). We genotyped the fish using restriction-site associated DNA (RAD) sequences or RAD-tags (RAD-seq) (Miller et al. 2007, Baird et al. 2008, Davey et al. 2011). RAD-seq is a genome complexity

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[32] For South Puget Sound populations undetected at NAR, CPS, ADM, and SJF; central Puget Sound populations, CPS, ADM, and SJF; and Hood Canal populations, HCB, ADM, and SJF. See Figure 1

reduction technique that sequences subsets of the genome that are adjacent to restriction enzyme recognition sites, and can characterize a genome-wide assessment of molecular diversity. Generally, RAD-seq can identify 1,000s to 10,000s SNPs. For this project we used the SbfI restriction enzyme. RAD-seq libraries were prepared at the WDFW's Molecular Genetics Laboratory, and sent to the University of Oregon Genomics Core Facility for sequencing using an Illumina HiSeq2000 sequencer.

We used the program STACKS (Catchen et al. 2011, Catchen et al. 2013) to identify homologous RAD-tags, to generate an initial list of SNPs, and to genotype all individuals at these SNP loci. We used two different sets of parameters in STACKS to establish two independent catalogs. We then selected RAD-tags that appeared in both catalogs, and aligned this combined and reduced catalog using the program BOWTIE 2 (Langmead and Salzberg 2012) to a third catalog developed by Sewall Young (WDFW, unpublished data). From this alignment we were able to place our RAD-tags on Young's unpublished linkage map, and to remove paralogous sequences. We ranked each SNP in a RAD-tag by the number of individuals genotyped at that SNP and by the frequency of the most common (i.e., major) allele, with the larger number of individuals genotyped and the lower frequency of the major allele receiving the higher score. We selected the one SNP with the highest ranking to represent the RAD-tag; in the event of a tie, we randomly selected a SNP from among the highest ranking SNPs. Finally, we eliminated all SNPs with a minor allele frequency less than 0.05, producing a total of 8598 from STACKS.

We continued to examine the dataset following STACKS to generate a relatively simple first-attempt at associating SNP genotypes with fate. To this end, we eliminated all samples that had fewer than 80% of the SNP loci scored (n=13), or were not identified unambiguously as a survivor or mortality (49), reducing the dataset to 226 individuals (Table 1). Next, based on principal component analysis (PCA) we eliminated outlier sets individuals (Figure 2). Finally, in terms of samples, we removed populations with either low overall sample sizes or extreme differences in the number of survivors and mortalities. However, we retained all samples from the 2014 Green and Nisqually river collections, as these fish were involved in a reciprocal translocation experiment testing the relationship between release location and survival while controlling source location. Therefore, our final dataset included only collects from the Skokomish, Green, and Nisqually rivers; 104 individuals (70 mortalities, 34 survivors) out of the original 288 individuals that were RAD sequenced (Table 1). Finally, we increased the minor allele frequency (MAF) threshold removing all SNPs with MAF less than 0.10, producing a final dataset consisting of 104 individuals and 5702 SNP loci.

We used the mixed linear model (MLM) procedure in the program TASSEL (Yu et al. 2006, Bradbury et al. 2007, Zhang et al. 2010) to provide a preliminary test for associations between fate and genotype. Simply, the MLM attempts to solve: phenotype = genotypes + population structure + family structure (kinship) + residual, with genotypes and population structure being fixed effects and kinship and residuals being random effects. Phenotype is fate (survival or mortalities) plus factors (smolt migration year, source location, and release location; see below). We used the program STRUCTURE, with admixture (Pritchard et al. 2000, Falush et al. 2003, Hubisz et al. 2009), to determine population structure of the three source locations using a reduced dataset consisting of 1043 SNP loci (i.e., all loci that were scored in all 104 individuals). The analysis with K = 4 groups provided the highest likelihood. Q-scores for each individual across the four groups (covariates) sum to 100%, which if all four covariates are included in the analysis will create linear dependency among the covariates. Therefore, as recommended in the TASSEL manual, to prevent the linear dependency we removed the Q-scores for the fourth group or covariate. Pairwise kinship between each pair of individuals was calculated in TASSEL, which calculates kinships as a scaled identity-by-state distance. Finally, we implemented six MLMs each with a different phenotype state (fate + factors): (1) fate only (no factors); (2) Fate + smolt migration year (Year); (3) Fate + smolt source location (Source); (4) Fate + smolt release location (Release); (5) Fate + Year + Source; and (6) Fate + Year + Release. We defined a significance association between a SNP locus and fate visually using quantile-quantile (QQ) plots (locus deviation off of straight line; Figure 3), and as probability ≤ 0.05, adjusted for false discovery rate (FDR; Hochberg and Benjamini 1990, Benjamini and Hochberg 1995). Fate is a categorical phenotype; since TASSEL assumes that the phenotype is a quantitative trait the probability associated for each SNP will be biased low (i.e., showing greater significance).

There was only one SNP locus that was significant or nearly significant in all MLMs: 39529_18 (Figure 3, Table 2). Three other loci were significant for the Fate + Year + Release MLM only: 55970_7, 12301_21, and 51226_71 (Figure 3, Table 2). We used the Basic Local Alignment Tool (BLAST) at NIH's National Center for Biotechnology Information (NCBI) website to match the RAD-tag from each of these four loci to sequences of known identity. The quality of match between the RAD-tag and sequences in the NCBI database is determined by the match's expected-value (E-value); the lower the E-value the higher the confidence in the match. We did not consider matches with E-value greater than 1×10^{-5} (1e-5). The RAD-tags are 80 basepair (bp) long, which is relatively short and resulted in multiple matches for each of the four SNP loci (Table 2). The number of qualifying matches ranged from zero (55970_7) to 14 (51226_71). Across all four loci, the match with the lowest E-value (6e-24) was between 39529_18 and sequences linked to Hox gene clusters in Atlantic salmon. Hox genes control morphogenesis along the anterior-posterior axis, and can be involved with limb (fin) development (Schneider et al. 2011, Pascual-Anaya et al. 2013, Schneider and Shubin 2013, Freitas et al. 2014). Although the match between 39529_18 and the Hox gene cluster was nearly perfect, it occurred in a non-coding part of the Hox gene cluster. 51226_71 matched with 14 different sequences with E-values greater than 1e-5, 13 matching Atlantic salmon sequences, and one matching a rainbow trout/steelhead sequence. The match with rainbow trout/steelhead had the second lowest E-value (2e-13) for this RAD-tag and involved an immunological gene. The match with the lowest E-value (6e-14) was to a sequence linked to Hox gene clusters in Atlantic salmon. In total nearly half of the matches for 51226_71 involved immunological genes (Table 2). As with the 39529_18 matches, none of the matches with 51226_71 occurred in a coding part of the gene. The two matches with 55970_7 had E-values greater than 1e-5 and therefore these sequences did not qualify as significant matches. However, both matches were to Atlantic salmon genes, one involving an immunological gene, and the other a regulatory gene. Finally, none of the five sequences that matched to 12301-21 involved salmonid sequences, and were genomic sequences of unknown function.

This dataset lacked power to provide a definitive association between smolt genotypes and fate: (1) sample sizes were too small and post-hoc test were not possible; (2) there was a lack of independence between year and source and between source and release; (3) fate is a categorical phenotype while the model was built for quantitative data; and (4) the RAD-tag sequences were too short to match more specifically with known sequences in the NCBI database. Nevertheless, there are at least two findings from our analysis that are worth pursuing with additional analyses. First, the HOX gene match with 39529_18 and 51226_71 loci suggests that there may be a developmental difference between survivors and mortalities in morphological features that may be involved with swimming performance (axial and fin development). Second, matches involving immunological genes suggest that there may be a difference between survivors and mortalities in how individual fish respond to pathogens or parasites.

Table 1. Number of samples from each source location and collection year that were RAD sequenced (Complete), had unambiguous fate (mortality or survivor) and sufficient number of loci genotyped (Intermediate), and were included in the final analysis (Final; see text).

Source Location	Complete			Intermediate		Final	
Collection Year	Mortality	Survivor	Ambiguous	Mortality	Survivor	Mortality	Survivor
Big Beef Creek							
2006	8	17	3	8	17	0	0
2007	7	6	0	7	6	0	0
2008	5	3	2	5	2	0	0
2009	3	1	2	2	1	0	0
2010	7	2	3	5	2	0	0
Dewatto							
2006	2	2	0	2	2	0	0
2007	11	2	1	10	2	0	0
Duckabush							
2009	10	3	5	10	3	0	0
Hamma Hamma							
2006	6	6	0	6	6	0	0
2007	4	4	0	4	4	0	0
Skokomish							
2006	6	6	0	5	6	5	6
2007	6	5	0	5	5	5	5
2008	15	5	10	14	5	8	4
2009	6	4	2	6	3	4	3
2010	16	0	0	15	0	7	0
Green							
2008	7	3	4	7	2	7	2
2014	22	9	10	20	9	20	9
Nisqually							
2014	15	5	7	15	5	14	5
TOTAL	156	83	49	146	80	70	34

Table 2. Top: Probabilities for the null hypothesis of no association between SNP locus and phenotype (fate + factor) for the four loci significant at least at one MLM. Probabilities in bold typeface were significant at alpha = 0.05 adjusted for false discovery rate (FDR). Bottom: Total number of sequences in the NCBI database that matched the 80 bp RAD-tag for each locus at E-values ≤ 1e-5 (N), and of those sequences the number whose function can be classified as morphogenesis, immunological, or other.

Phenotype	Locus			
(MLM)	39529_18	55970_7	12301_21	51226_71
Fate	1.54E-05	3.49E-04	5.07E-01	9.14E-02
Fate + Year	**2.92E-06**	2.16E-03	3.96E-01	1.40E-01
Fate + Source	1.42E-05	2.80E-04	4.74E-01	1.51E-01
Fate + Release	1.46E-05	2.63E-04	5.00E-01	1.40E-01
Fate + Year + Source	**3.97E-06**	2.53E-03	3.74E-01	1.21E-01
Fate + Year + Release	**4.18E-06**	**3.62E-158**	**6.63E-117**	**9.44E-83**
N	2	0	5	14
Morphogenesis	1	0	0	1
Immunological	0	0	0	6
Other	1	0	5	7

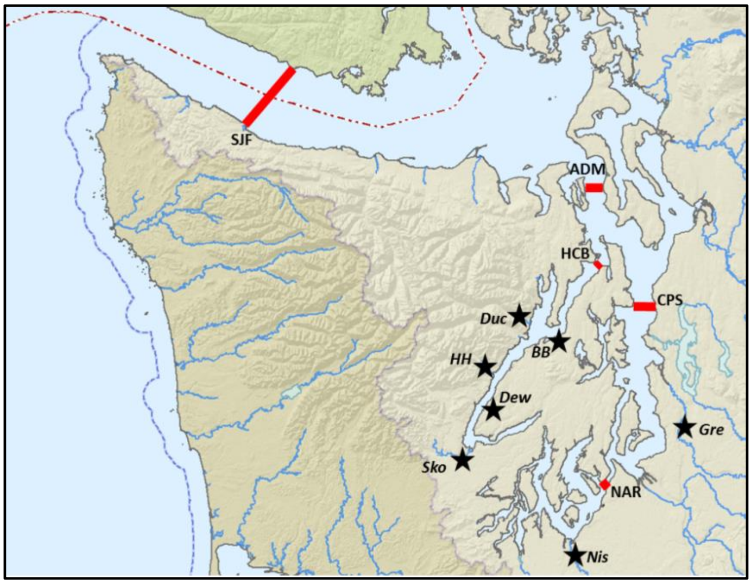


Figure 1. Location of the acoustic receivers (red lines), and general source location of the samples (black stars). Abbreviations: Receivers – Strait of Juan de Fuca (SJF), Admiralty Inlet (ADM), Hood Canal Bridge (HCB), Central Puget Sound (CPS), Tacoma Narrows (NAR); Source locations –Big Beef Cr (BB), Dewatto R (Dew), Duckabush R (Duc), Hamma Hamma R (HH), Skokomish R (Sko), Green R (Gre), and Nisqually R (Nis).

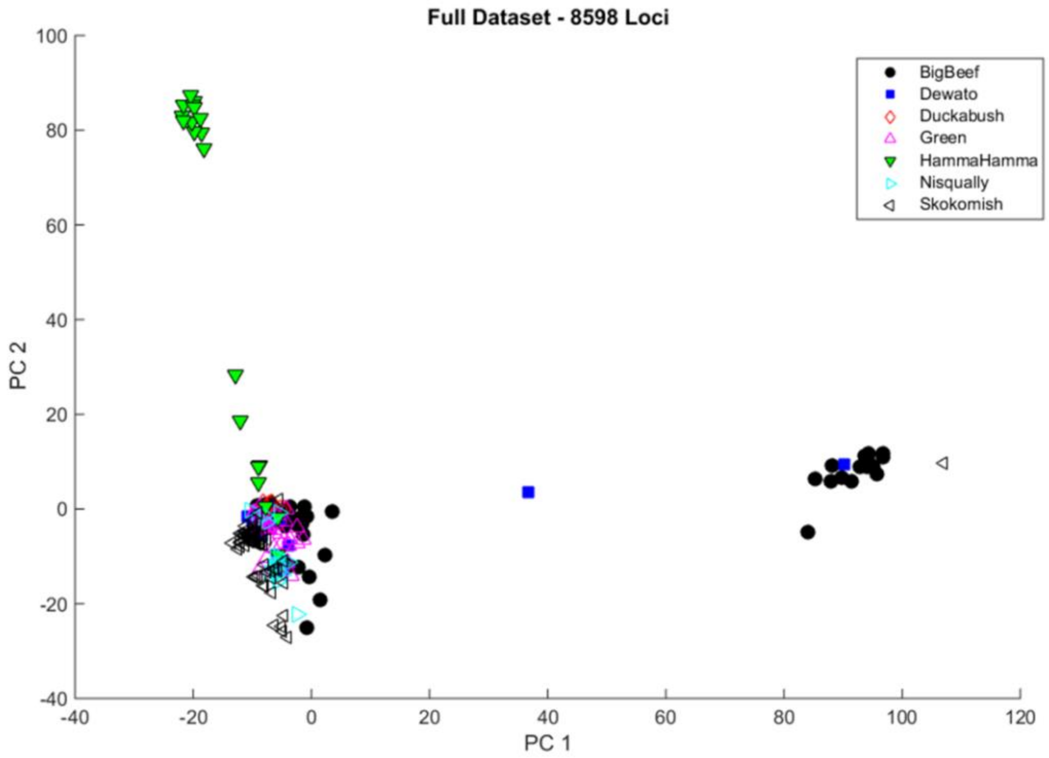


Figure 2. Principal component analysis of all samples using the full 8598 SNP locus data set. Outlier sets of individuals are those that differ from the core set at the lower left of the plot: Big Beef, Dewato, and a single individual from Skokomish along the PC1 axis, and Hamma Hamma along the PC2 axis.

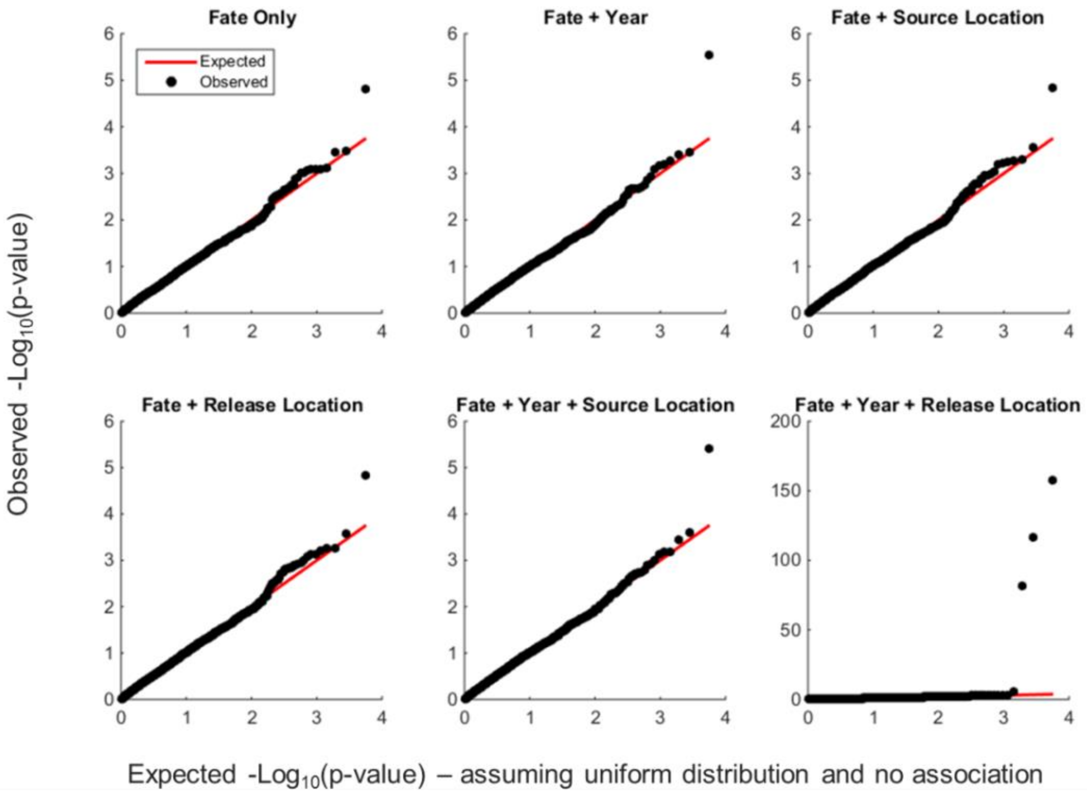


Figure 3. Log quantile-quantile (QQ) probability plots for each of the six MLM analyses. Each filled circle represents a SNP locus (5702 loci in each plot). The red line represents the expected distribution, or null hypothesis of no association between the SNP and fate plus factors. SNPs that appear at a distance from the line indicate a significant association between that SNP and fate plus factors. The one SNP that appears significant in all plots is 39529_18. In the lower right plot, the significant SNPs, from right to left, are 55970_7, 12301_21, 51226_71, and 39529_18. Note differences in scale of y-axis between the plot in the lower right and all other plots.

Study 13: Genome-wide association study part 2: using (1) survival in acoustically tagged, and (2) Nanophyetus salmincola infested steelhead smolts in south/central Puget Sound, Washington

Kenneth I. Warheit[1], Megan E. Moore[2], Barry A. Berejikian[2]

[1]Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501-1091 USA
email: kenneth.warheit@dfw.wa.gov

[2]Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration Fisheries, P.O. Box 130, Manchester, 98353, USA

(*Technical Report: Warheit K.E.; M.E. Moore, B.A. Berejikian. 2018. Genome-wide association study of acoustically tagged steelhead smolts in the Salish Sea: measuring differences between survivors and non-survivors. Washington Department of Fish and Wildlife, Fisheries Division, Olympia. Available at www.marinesurvivalproject.com/resources*)

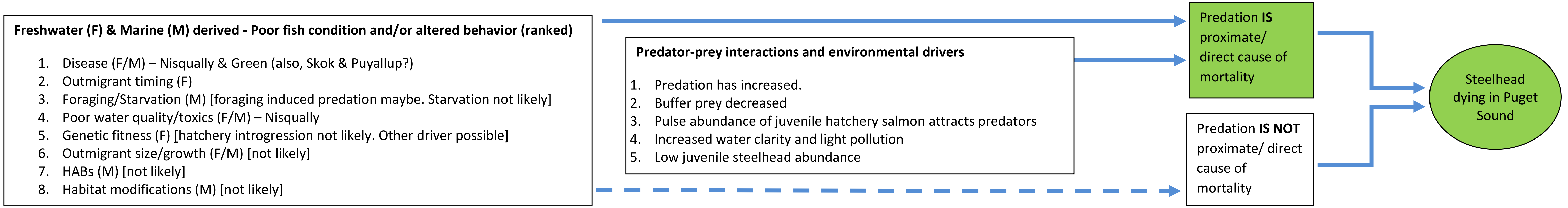
The purpose of this study was to test the hypothesis that there is a genomic association with (1) survival of outmigrating steelhead smolts as they transit from either the Green or Nisqually rivers through Puget Sound to the Pacific Ocean, or (2) *Nanophyetus salmincola* infestation in steelhead smolts captured in the freshwater, estuary, or offshore areas on the Green or Nisqually rivers. In this project's pilot study (Warheit et al. 2015), we attempted to test for genomic associations with smolt survival in acoustically tagged fish from Big Beef Creek, and Dewatto, Duckabush, Hamma Hamma, Skokomish, Green, and Nisqually rivers in Puget Sound, Washington. Collections were stratified by collection year, location, lineage, which was hierarchical, and release location, and survival was defined as a binary phenotype (survived to or did not survive to western Strait of Juan de Fuca). The many rivers and therefore lineages and collection years, and the few individuals that were categorized as survived, created small samples sizes and limited statistical power. For this current study we attempted to improve the pilot study's sample design by limiting the analysis to the Green and Nisqually rivers collected in 2014 as part of the reciprocal translocation project, and to the 2015 Nisqually River samples. Therefore, for this project we genotyped all samples from 2014 and 2015 from the Green and Nisqually rivers. In addition, we added an additional data set for the *Nanophyetus salmincola* infestation study. From both the survival and *Nanophyetus* data sets, we conclude that there is a genomic association with both steelhead smolt survival and *Nanophyetus* infestation, but the association is statistically weak.

For both the acoustic-survival and the *Nanophyetus* data sets, the Omy05 genotypes were associated with survival-index and *Nanophyetus* counts. The Omy05 genotypes represent a large linkage group associated with a chromosomal inversion; the genotypes are maintained by the absence of recombination (Pearse et al. 2014). In the California rivers included in Pearse et al. (2014), resident fish above migration barriers most commonly possess the A allele, while the anadromous fish below the barriers have the R allele (Pearse et al. 2014, Y. Palti, personal communication 2017 for allele nomenclature). We do not know if the Omy05 genotypes in Puget Sound are also associated with juvenile migration life histories. All fish used in this study were smolts, all of whom we assumed would eventually migrate to the ocean if they hadn't died in route (acoustic-survival) or euthanized for the *Nanophyetus* study. If the Omy05 genotypes here are associated with migration life histories, it is possible that the Omy05 A allele is maintained in the anadromous steelhead population by resident rainbow trout, and the presence of that A allele may reduce the individual's probability of survival or will result in a higher *Nanophyetus* count, which directly or indirectly may reduce survival.

Other components of the genome are more difficult to discern. In the acoustic-survival data set there were a total of 13 significant loci, across three different analyses, located on at least nine chromosomes (2 loci were unmapped), and of which only six loci have been identified with possible functions. Furthermore, seven of these 13 loci are only significant in fish released from the Nisqually River (one locus, 68900_81 was significant in both the Nisqually R. release and the 2014 all samples data sets). Two of the eight loci significant in the Nisqually R. release data set may be related to circadian clock, and another locus with the immune system. The circadian clock loci may be associated with daily components of migration timing, which may be related to survival. Since these loci appear important only in fish released from the Nisqually River, they may be important only for the migration segment not in common with the Green River, or for fish that have a longer migration distance. There are many loci significantly associated with *Nanophyetus* counts, but the pattern of significance differs between the Nisqually and Green river populations, which have different prevalence of *Nanophyetus* (study 10).

No definitive conclusion can be drawn from this study. Despite an increase in sample sizes from the pilot study, for most analyses the study lacked sufficient sample sizes to achieve sufficient statistical power. How far a wild steelhead smolt can migrate through Puget Sound or how many *Nanophyetus* that smolt contracts is not independent of the smolt's genome. However, this study did not clarify the relative importance of the genome, compared with environmental factors, or how the genome interacts with these environmental factors to affect survival or prevalence of *Nanophyetus*. Adding genome-association and functional genomic components to any experimental study that relates smolt performance with the prevalence of *Nanophyetus* will contribute to understanding the relationship of the genome to smolt survival.

APPENDIX B: LOGIC MODEL CROSSWALK WITH 2013-2017 RESEARCH FINDINGS



Freshwater (F) & Marine (M) derived - Poor fish condition and/or altered behavior (ranked)

1. Disease (F/M) – Nisqually & Green (also, Skok & Puyallup?)
2. Outmigrant timing (F)
3. Foraging/Starvation (M) [foraging induced predation maybe. Starvation not likely]
4. Poor water quality/toxics (F/M) – Nisqually
5. Genetic fitness (F) [hatchery introgression not likely. Other driver possible]
6. Outmigrant size/growth (F/M) [not likely]
7. HABs (M) [not likely]
8. Habitat modifications (M) [not likely]

Predator-prey interactions and environmental drivers

1. Predation has increased.
2. Buffer prey decreased
3. Pulse abundance of juvenile hatchery salmon attracts predators
4. Increased water clarity and light pollution
5. Low juvenile steelhead abundance

Predation **IS** proximate/ direct cause of mortality

Predation **IS NOT** proximate/ direct cause of mortality

Steelhead dying in Puget Sound

The ultimate source of mortality in Central and South Puget Sound is likely marine derived and not associated with freshwater habitat or hatchery influence. However, causes derived in the lower river, or fish condition effects consistent among steelhead populations, cannot be ruled out. – reciprocal transplant

Evidence/Findings:

- *Nanophyetus salmincola*, with new infections occurring in the lower river, may kill outmigrating steelhead or make these juvenile steelhead more vulnerable to predation, contributing to lower early marine survival rates of steelhead populations in Central and South Puget Sound.
- PBDE's, a contaminant, may affect the health of steelhead leaving the Nisqually River; however, its impact may depend upon the rate it can affect steelhead in the lower river. The other contaminants analyzed and for the Nisqually, Green, and Skagit were less of a concern; however, PCBs increased above adverse effects thresholds in samples taken from steelhead collected offshore in Puget Sound.
- Smolts in some populations with particular genetic fingerprints may be predisposed to higher early marine mortality and higher N. salmincola loads. This may be associated with the influence of residency vs anadromy. In some cases, the circadian clock and immune system may also influence parasite loads and survival. However, the power of these findings is currently limited. (Nisqually and Green studied)
- In low early marine survival years, juvenile steelhead migrating in April and late May survive at higher rates than steelhead migrating in early-mid May. While not yet investigated, this may be associated with factors such as changes in predator-prey dynamics or N. salmincola shedding events/disease outbreaks.
- A steelhead foraging-predation rate relationship was not investigated, but starvation is not likely.
- Whole body lipid content was less than 1.5% in the wild steelhead populations that were assessed (Skagit, Green, Nisqually Low lipid levels are not inconsistent with a decline in whole body lipid content toward depletion during the smolt outmigrant life-stage. However, levels below 1% (in some steelhead) may be cause for concern as 1% has been documented as a threshold for the onset of high over-winter mortality in rainbow trout.
- Juvenile steelhead size at outmigration and steelhead outmigrant abundance are not correlated with survival among years. Size at outmigration is also not correlated with survival within years.

Evidence/Findings:

- An increase in the abundance of harbor seals correlates with the decline in steelhead.
- Abundance trend data are lacking for a correlative assessment with other potential predators.
- Given the significant increase in abundance/prevalence of harbor porpoise, the potential impact should be investigated.
- The presence of alternative or "buffer" prey, in high abundance, may improve steelhead survival
- A decline in the abundance of hatchery Chinook, combined with more consolidated release timing of hatchery Chinook subyearlings, may affect predator behavior and make steelhead more vulnerable to predation.
- The presence of transient killer whales may impact harbor seal and harbor porpoise behavior and abundance
- Increased water clarity and light pollution may exacerbate predation; however, paucity of data limits analyses
- Other environmental drivers including Puget Sound sea-surface temperatures and the North Pacific Index may contribute to the factors affecting overall marine survival.

Evidence/Findings:

- Steelhead are dying at rapid rates, most within 10 days (likely excludes starvation, & possibly disease, toxics).
- Mortality not highly variable among years (likely excludes HABs, etc)
- The list of most likely, potential bird and marine mammal predators of outmigrating juvenile steelhead includes harbor seals, harbor porpoises, double-crested cormorants, Caspian terns, and Brandt's cormorants.
- Harbor seals are a source of proximate mortality in South and Central Puget Sound.
- Nano-saltwater challenge did not result in direct mortality; however, nano infection was not new.
- Of those contaminants investigated (Total PCBs, Σ_{11}PBDEs, Σ_{6} DDTs, HCB, Σ_{8}Chlordanes, Σ_{3}HCHs, Σ_{37} PAHs, and estrogenic chemicals) the levels are not high enough to suggest direct mortality.

Evidence/Findings:

- Puget Sound steelhead population abundance and marine survival have declined and remain lower than other nearby regions.
- Puget Sound steelhead early marine survival rates are low, with the highest instantaneous mortality rates in South and Central Puget Sound, and the north end of Hood Canal through Admiralty Inlet. Early marine survival increased in 2016, with the greatest reduction in mortality occurring in Central Puget Sound
- Typically, the farther steelhead must swim through Puget Sound, the greater the mortality (death by distance traveled).