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Title: Cool, Pathogen Free Refuge Lowers Pathogen Associated Prespawn Mortality of Willamette River Chinook Salmon (*Oncorhynchus tshawytscha*).

Abstract approved: _____

Carl B. Schreck

Spring Chinook salmon, *Oncorhynchus tshawytscha*, are transported above dams in the Willamette River to provide access to blocked spawning habitat. However, 30-95% of these transplants may die before spawning in some years. To varying degrees, salmon in other tributaries--both blocked and unblocked-- have similar prespawn mortality (PSM) . Our study determined if holding in constant temperature, pathogen free conditions prior to spawning increased survival to spawn. In addition, we evaluated pathogens as a potential cause of PSM.

Adult Chinook were captured early and late in the season from the lower Willamette River and from upper river tributaries and held in constant, cool temperature (13 ˚C), pathogen-free water at Oregon State University. Additional fish were sampled at time of transport from each of the collection sites. Finally, recent mortalities were collected from river surveys on holding and spawning reaches above traps. Necropsies were performed on all fish, and samples were processed for histology. Held fish were spawned to determine if progeny were viable.

Held fish were less likely to be a PSM than fish that were outplanted to the river. However, bacterial infections were more prevalent in held fish than outplanted fish. Consistent with these observations, PSM in held fish was more likely to have higher

burdens of pathogens than spawned fish. Held spawned fish were more likely to have *Myxobolus* sp. brain infections and less likely to be infected with the kidney myxozoan *Parvicapusla minibicornis* than spawned outplanted fish. The equal likelihood of other pathogens for held and outplanted spawned fish suggests interactive effects determine survival and that holding at 13 ˚C prevented expression of lethal pathogenesis. Progeny of held fish from all locations and collection dates were viable. Overall, holding could be a viable method to reduce PSM, but issues of transport stress, proliferative disease such as those caused by bacteria, and antibiotics remain.

Cool, Pathogen Free Refuge Lowers Pathogen Associated Prespawn Mortality of Willamette River Chinook Salmon (*Oncorhynchus tshawytscha*).

by
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APPROVED:

Major Professor, representing Fisheries Science

Head of the Department of Fisheries and Wildlife

Dean of the Graduate School

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

Susan Benda, Author

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CONTRIBUTION OF AUTHORS

Dr. Carl Schreck, Dr. Michael Kent and Dr. Chris Caudill were co-principle investigators and involved in all aspects of this study: design, data collection and key concepts. Dr. Michael Kent read and scored slides for prevalence and severity of pathogens. Dr. Chris Caudill facilitated transport and collection of fish, and helped obtain PSMs. Cameron Sharpe assisted with data collection, fish transport, and organized crews to get PSM's.

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Abstract

Spring Chinook salmon, *Oncorhynchus tshawytscha*, are transported above dams in the Willamette River to provide access to blocked spawning habitat. However, 30-95% of these transplants may die before spawning in some years. To varying degrees, salmon in other tributaries--both blocked and unblocked-- have similar prespawn mortality (PSM). Our study determined if holding in constant temperature, pathogen free conditions prior to spawning increased survival to spawn in 2010 through 2012. In addition, we evaluated pathogens as a potential cause of PSM.

Adult Chinook were captured early and late in the season from the lower Willamette River and from upper river tributaries and held in constant, cool temperature (13 ˚C), pathogen-free water at Oregon State University. Additional fish were sampled at time of transport from each of the collection sites. Finally, recent mortalities were collected from river surveys on holding and spawning reaches above traps. Necropsies were performed on all fish, and samples were processed for histopathological analysis. Held fish were spawned to determine if progeny were viable.

Held fish were less likely to be a PSM than fish that were outplanted to the river. However, bacterial infections were more prevalent in held fish than outplanted fish. Consistent with these observations, PSM in held fish was more likely to have higher burdens of pathogens than spawned fish. Held spawned fish were more likely to have *Myxobolus* sp. brain infections and less likely to be infected with the kidney myxozoan *Parvicapusla minibicornis* than spawned outplanted fish. The equal likelihood of other pathogens for held and outplanted spawned fish suggests interactive effects determine survival and that holding at 13 ˚C prevented expression of lethal pathogenesis. Progeny of held fish from all locations and collection dates were viable. Overall, holding could be a viable method to reduce PSM, but issues of transport stress, proliferative disease such as those caused by bacteria, and antibiotics remain.

Introduction

The historic rates of prespawn mortality (PSM), defined by us to be mortalities that occur after migration to spawning grounds but prior to ovulation or spermiation, are not well documented in salmon. Given the physiological and environmental challenges of migration, it is natural for some death to occur before spawning (Gauthreaux 1980; Quinn 2005). However, the chance of a self sustaining population decreases with as little as 10% PSM (Keefer et al. 2010) and PSM in the upper Willamette River has reached up to 90% (Schroeder et al. 2007).

The Willamette River, Oregon, population of spring Chinook salmon are listed under the U.S. Endangered Species Act and natural populations are largely supplemented by hatchery stock (NMFS 2008). Dams contribute to their decline, given dam effects on passage, temperature, and flow (NMFS 1999). In an effort to both create self sustaining runs and provide access to dam blocked spawning habitat, fish are transported above dams into upstream tributaries (these fish are hereafter referred to as outplanted fish). Transporting fish (both adults and juveniles) is not an unusual practice (Zimmerman and Duke 1995; Engle and Skalicky 2009; Mosser et al. 2013), and it is important to note that PSM occurs in both transported and non transported populations. Therefore, with the goal of effectively managing salmon populations, it is necessary to both determine the cause of prespawn mortality and evaluate potential management tactics.

Pacific salmon are exposed to a suite of proliferative and non- proliferative freshwater pathogens during their migration and holding on spawning grounds. We define proliferative pathogens as those that multiply within the fish host, whereas non-proliferative do not. Concurrently, Pacific salmon experience high levels of cortisol, which is known to be an immunosuppressant (Schreck 1996). Thus, normally innocuous pathogens have the potential to dominate. As a result, death after spawning is generally from disease or energy depletion (Schreck et al. 2001).

Pathogens have also been associated with PSM, e.g. *Parvicapsula minibicornis* in sockeye salmon (*Oncorhynchus nerka*) of the Fraser River (Raverty et al. 2000) or ichthyophoniasis in Chinook salmon of the Yukon river (Kocan et al. 2004). Adult spring Chinook salmon in the Willamette River can be infected with both proliferative*(Ceratomyxa shasta,* a *Myxobolus sp.* of the central nervous system, *Parvicapsula minibicornis, Renibacterum salmoninarum, Aeromonas*

*salmonicida),*and non proliferative (*Nanophyetus salmincola, Apophallus* species and *Echinochasmus milvi)* pathogens (Kent et al. 2013). One of the hypotheses this study sought to answer was whether pathogens are correlated with PSM in spring Chinook salmon of the Willamette River.

Elevated temperature is another environmental variable that correlates with prespawn mortality (Crossin et al. 2008; Keefer et al. 2010; Jeffries et al. 2012). Temperatures outside the acceptable range can cause a change in energy requirements for basal metabolic activity (McCullough 1999). This leads to a depletion of energy that could have been used for migration, reproduction, and immune responses (McCullough 1999). Temperature can also create a more suitable environment for pathogens, either through increasing host susceptibility, increasing transmission rates, or shortening temperature dependent life cycles (Rucker et al. 1954). Furthermore, as temperature is affecting spawning adults, it has an inherent effect on progeny survival (McCullough 1999; Beer & Anderson 2001; Quinn 2005).

The primary focus of this study was to determine if salmon held in a pathogen free, constant temperature environment have lower PSM than those in the river. We hypothesized that fish held in a constant cool temperature facility with no pathogen exposure while being held in captivity would have higher survival to spawn than outplanted fish (Hypothesis H1). Of particular interest was the effect of length of holding on adult survival as this would influence future management recommendations. We also hypothesized that fish taken earlier from the river, (both spatially and temporally), will have had less exposure to the river environment and perhaps fewer pathogens and may have a higher survival to spawn than fish taken later (H2).

The second aim of this study was to examine the suite of pathogens infecting adult salmon under different conditions (e.g., held vs outplants, PSM vs live fish sampled at trapping facilities, and PSM vs spawners collected in the fall). Histology was chosen because it allowed examination at the tissue level for known and unknown pathogens (Kent et al. 2013). We expect spawned fish to have higher burdens than fish earlier in the season, due to the longer exposure to pathogens, increased susceptibility with senescence and because fish die from pathogens after spawning. Therefore, if prespawn fish are dying due to pathogen associated mortality, we predicted that

they would have similar pathogen burdens as a successfully spawned fish (H3). When we compare outplanted fish and fish at the holding facility, we expect to find lower pathogen burdens for fish at the latter, because they are removed from pathogen exposure and because we expected they would have lower thermal loads (H4).

Methods

Figure 1. Map of the Willamette River. Willamette Falls, Foster, Fall Creek and Dexter traps are starred, the Fish Performance and Genetics Laboratory is marked by a triangle. The insert is the

location of the Willamette basin in the State of Oregon. (source: http://greenbeltlandtrust.files.wordpress.com/2010/01/willamette-basin.gif)

Field Sites and Holding facility:

Adult hatchery-origin salmon were collected from as low in the system as possible, which was at Willamette Falls, and then in spawning tributaries upstream at Dexter Dam, Fall Creek Dam, and Foster Dam (Figure 1). In addition, carcasses were collected from tributaries above the traps on the North Fork Middle Fork of the Willamette River, Fall Creek, and South Santiam River, respectively.

Fish were transported to the Fish Performance and Genetics Laboratory (hereafter referred to as the holding facility) of Oregon State University (OSU) located in Corvallis, Oregon for holding in cool, pathogen free water. This facility uses pathogen free well water, and the flow through system ensures a constant temperature of 13 ˚C ($\pm$1 ˚C seasonal variation). Fish were held in six outdoor tanks (measuring 3 meters in diameter with a water depth of 1 M). Tanks were covered with black screen to provide shade and prevent escapes.

All work was done in conformance with OSU Institutional Animal Care and Use Committee (ACUP 4438). The use of AQUI-S 20E was conducted under INAD protocol 11-741 (from USFWS-AADAP). Adult salmon were sampled under Endangered Species Act Take permits W1-10-UI200, W1-11-UI200, W1-12-UI200 issued by NOAA-Fisheries and appropriate state scientific collection permits issued by ODFW.

Transport & holding:

Adult salmon from collection sites mentioned above were transported to the holding facility. Procedures at each trapping facility varied slightly and are described below.

At Willamette Falls, fish were diverted from the fish ladder into a trap. Fish had to voluntarily exit through a Denil fish ladder where they slid into an anesthetic bath. In 2011, fish were anesthestized using 50 mg/L tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate to a pH of 7.0. AQUI-S 20E® at a dose of 22 ppm was used in 2012. Fish were either sampled on site for later parasite assessment or transported. For transport, anesthetized

fish were placed into individual cylinders filled with oxygenated water, then moved to a transport truck located about 15 minutes away. We waited up to three hours after the collection of the first fish to allow for the collection of as many fish as possible before fish were trucked to the holding facility. This duration was selected to avoid holding mortality.

Fish returning to Dexter and Foster dams ascended a fish ladder and entered raceways where they were held for a period of up to several days. They were then crowded into a carbon dioxide (CO_2) bath that allowed the fish to be handled and lifted to a processing table. From here they were either euthanized for necropsy or individually netted into a transport truck.

At Fall Creek, fish were crowded from the ladder into a holding tank and anesthetized with AQUI-S 20E®. They were then sampled or individually netted into a transport truck.

Fish recovered from the anesthetic in the transport vehicles. It took about 2 hours to transport fish from Willamette Falls, Dexter and Fall Creek traps and 1 hour to transport fish from Foster trap. Fish were transported by Army Corps of Engineers and ODFW in various transport tanks or trucks ranging from1.89 m^3 to 5.68 m^3. In all cases water was used from either the holding facility or the collection site (with a temperature of 13-15 ˚C) and was oxygenated throughout the transport period. Upon arrival at the holding facility the fish were anesthetized with buffered MS-222 to minimize effects of netting stress and then stocked into holding tanks.

Transport dates and sample sizes for each location and year are listed in table 1. Due to permit restrictions, we were not able to transport fish from Willamette Falls in 2010. While we aimed for two collection times per site, Willamette Falls required three. The run at Fall Creek is primarily natural origin fish, this tributary was not be sampled after 2010. Foster was added in 2012 as an additional location where fish are transported above a dam.

We attempted to obtain fish over a time period that spanned the timing of the run as closely as feasible. Fish enter freshwater around December, pass Willamette Falls in mid-March to July, and arrive at upper tributaries April through September.

Table 1. Transport dates to holding facility and respective sample sizes (N) for each location and year.

Location	Year	Date	N
Willamette Falls	2011	18-May	2
		5-Jun	5
		7-Jun	8
	2012	16-May	6
		23-May	5
		13-Jun	9
Dexter	2010	8-Jun	10
		14-Jul	10
			10
	2011	26-May	10
			10
		20-Jul	10
			10
	2012	6-Jun	15
		3-Aug	15
Fall Creek	2010	7-Jun	10
		12-Jul	7
Foster	2012	5-Jun	15
		2-Aug	15

At the holding facility each transport group was placed into individual outdoor tanks. No more than 15 fish went into a tank, i.e. when 20 fish from one location were transported they were split into two tanks. Because we had more than 2 collection dates at Willamette Falls (and a limited number of tanks), these fish were pit tagged and split equally into two tanks.

With the exception of a few Dexter groups (see Table 1), we did not have replicate tanks for each location and collection date within a year. This was due in part to limited space at the holding facility (at most 6 tanks) and by the number of fish we were permitted to take. Additionally, we did not cohabitate fish from different locations or collection dates out of concern of pathogen transmission and potentially losing all of the fish to disease. Because of these restraints, we

repeated the study for multiple years (2010 to 2012). While data from multiple years are not true replicates, it did allow us to examine inter-annual variation.

Hydrogen peroxide was administered every two weeks as an external fungal treatment because we were interested in parasite infections that occurred prior to the fish arriving at our facility. Fish were checked at least twice a day (more often towards spawning season) for mortalities. Dead fish were bagged, put on ice, and stored in a cold room until necropsied no more than 18 hours later.

Spawning & progeny:

Starting in September fish were checked weekly. Ripe females were characterized by loose eggs and ripe males by expression of sperm that dispersed quickly in water. Ripe females were euthanized with a blow to the head and eggs were removed following standard hatchery procedures. Males were live-spawned until all females had spawned; milt was collected in Whirl paks®, oxygenated and kept on ice. All dead fish were stored as described above for necropsy.

Eggs were fertilized with milt from 2-3 males from the respective females' tanks. If no ripe males from a respective tank were present, males from the next closest group (e.g. a replicate tank, or the other collection date) were used. Between 500 -1000 fertilized eggs per female were placed into Heath trays. The trays received ambient flow through water (12-13 ˚C) and columns were covered with black plastic to limit light disturbance. Dead progeny were removed to prevent fungal domination.

River samples:

River surveys of outplanted fish conducted by the Oregon Department of Fish and Wildlife (ODFW) provided 'fresh' mortalities (i.e. a fish with pink gills), which were placed in plastic bags and put on ice for later necropsy. Spawned fish were also sampled at Willamette Hatchery every year in September. Willamette Hatchery is located above Dexter Dam (Figure 1). Table 2 provides information on sample size for outplanted fish collected from river surveys and spawned fish collected at Willamette Hatchery.

To provide a baseline of what pathogen burden was at each collection site, 15 fish were euthanized and sampled for parasite burden at each site each year. We attempted to obtain fish over a time period that spanned the timing of the run as closely as feasible (Table 3).

Table 2: Sample sizes for PSM and spawned fish outplanted above dams for each location and year.

Location	Year	PSM	Spawned
Fall Creek	2010	8	11
Dexter	2010	19	6
	2011	13	2
Foster	2012	3	6
Willamette Hatchery	2010	0	26
	2011	0	28
	2012	0	27

Table 3: Sample sizes (N) for fish sampled for pathogen burden at each collection site by year and month.

Location	Year	Date	N
Willamette Falls	2011	May	9
		June	7
		July	8
	2012	May	9
		June	6
Fall Creek	2010	June	5
		August	6
Foster	2012	June	7
		August	6
Dexter	2010	June	3
		July/Aug	6
	2011	May	7
		July	4
	2012	June	5
		August	2

Tissue Processing:

A complete necropsy was performed on each fish. External and internal conditions were noted, as well as fork length, sex, percent spawned (for females), fungus, and adipose fin clip. Pieces of

gill, kidney, spleen, heart, brain, pyloric ceca, lower intestine, and liver were stored in 10% buffered formalin. After fixing for at least seven days, tissues were trimmed to 5 mm and placed in cassettes by our laboratory. Cassettes were embedded and sectioned by our laboratory in 2010 and by Veterinary Diagnostic Laboratory at OSU in 2011 and 2012. Slides were stained with hematoxylin and eosin by our laboratory.

Michael Kent, Oregon State University, identified the parasites present in each tissue sample. For each parasite in each tissue, severity of infection was scored on a scale of 0 to 3 based on intensity of infection and changes in tissue structure as a result of infection (see Kent et al. 2013). A score of 0 indicated the pathogen was not detected and no anomalies were seen in tissue structure. In comparison, a score of 3 indicated the pathogen was detected in high numbers and severe tissue changes were noted as a result of pathogen. While the scale is numeric, it is truly a qualitative assessment of severity, i.e. the pathogen was not detected (0) or detected in low(1), medium (2)or high(3) severity of infection. Rather than testing for single pathogens, histology allowed detection of a suite of pathogens, namely: *Ceratomyxa shasta*, *Parvicapsula minibicornis*, *Nanophyetus salmincola*, *Renibacterium salmoninarum*, *Aeromonas salmonicida*, *Apophallus* sp., *Echinochasmus milvi*, and *Myxobolus* sp. (see Kent et al. 2013 for applicability of this method).

Statistical Analysis:

Percent PSM of outplanted fish was supplied by ODFW and was based only on females because it cannot be determined if a male has spawned. Therefore, comparisons of percent PSM between fish at the holding facility and outplanted fish were made using only females. Conversely, comparisons within the holding facility of percent PSM used both males and females. Multiple logistic regression was used to determine associations between PSM and year, length of holding and location. We were unable to examine interactions between location, year or length of holding because we did not have replicate tanks for each location and collection date within one year. Further, May and June were designated as “Early” and July and August were considered “Late” collection dates to gain more replicates per group; this allowed us to take advantage of having data covering three return years.

Analysis of pathogen burden included all fish that had a score (0 to 3) for each pathogen— fish missing data were omitted. The highest severity score was chosen for pathogens that were present in multiple tissues. This way each fish had one score per pathogen, e.g. if *N. salmincola* scored a 3 in the heart, a 2 in gill and a 2 in the kidney, the score for that pathogen was 3. As we are interested in describing our observations, we provide prevalence of infection for each severity score in appendix A.

We also assessed presence/absence prevalence of infection of pathogens in live, prespawn, and spawned fish using two separate logistic regression models in R core team (2012). The models also account for location, year and collection (early, late, or outplant). These models will be described further in the results section to allow for easier interpretation.

Results

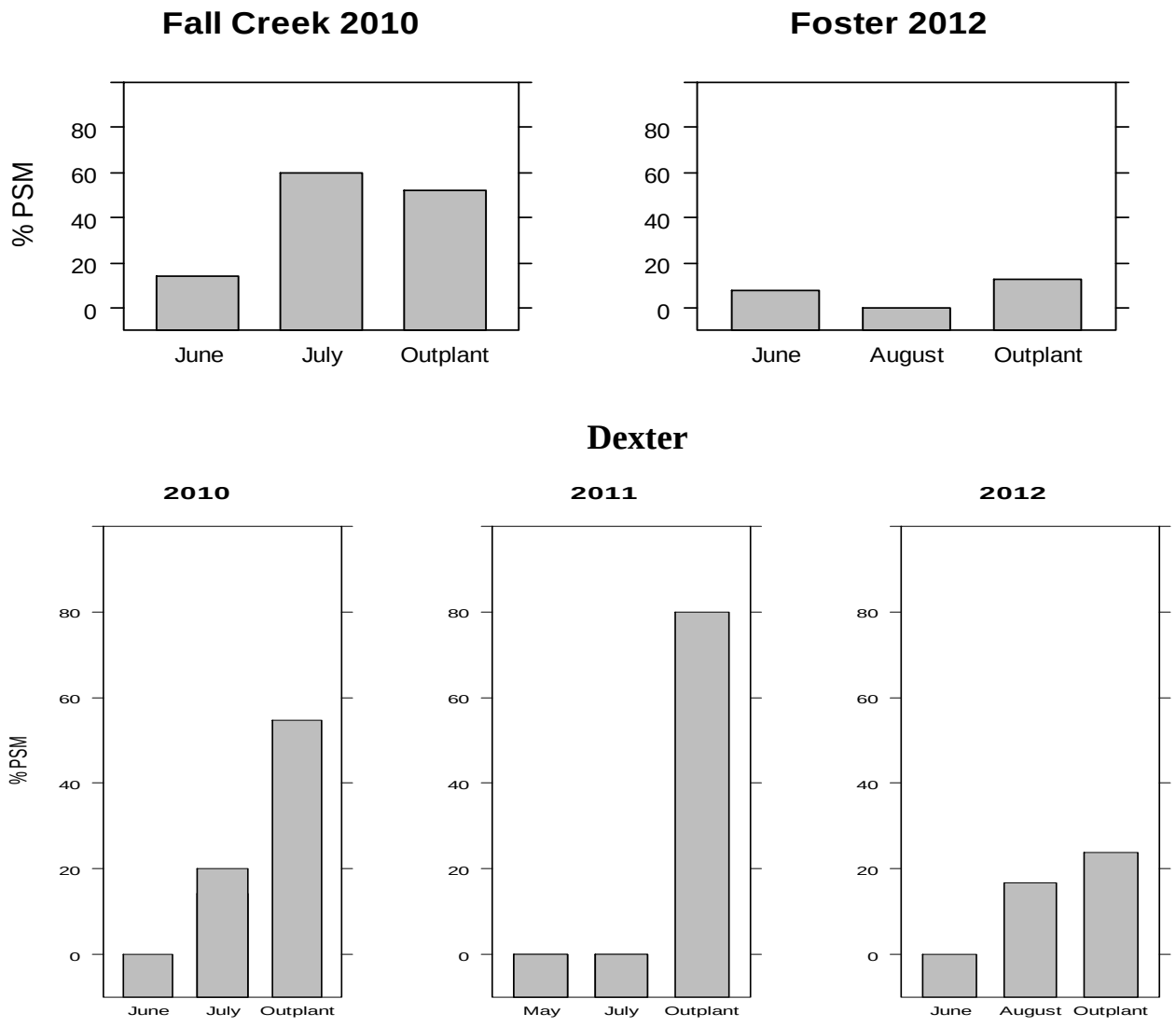

Figure 2. Percent prespawn mortality (PSM) for only female fish at the holding facility and outplanted above traps, for each location and year. Months indicate collection times for fish transported to the holding facility, and Outplant are fish outplanted above traps.

Table 4. Likelihood of PSM for female fish based on multiple logistic regression of collection groups, location, and year. The odds ratio applies to the first group in the comparison, e.g. PSM is 12.6 times more likely in the outplant group versus the early held group. Statistical significance indicated as follows: p< 0.001 '***'; 0.01'**'; 0.05'*'; 0.1'.'

Comparison	Odds Ratio (95% CI)		
Outplant v. Early	12.6	(3.4-81.4)	***
Outplant v. Late	7.9	(3.3-22.6)	***
Fall Creek v. Foster	2.3	(1.0-5.6)	.
Fall Creek v. Dexter	1.1	(0.6-2.0)	
2010 v. 2011	0.5	(03-1.1)	.
2010 v. 2012	3.8	(2.5-5.9)	***

Figure 2 depicts percent prespawn mortality (PSM) for only female fish at the holding facility and outplanted above traps, for each location and year. Using multiple logistic regression, we modeled the odds of PSM as a function of collection groups (early held, late held, and outplant), location (Foster, Fall Creek and Dexter), and year (2010-2012) for female fish only (Table 4). With this model, PSM was 12.6 times more likely in outplanted fish than Early collected held fish (95% CI 3.4 to 81.4 times), after accounting for location and year. It was 7.94 times more likely in outplanted fish than late collected held fish (95% CI 3.3 to 22.6 times). Fall Creek fish were 2.3 times more likely to have PSM than the Foster location (95% CI 1.0 to 5.6 times). Finally PSM in outplanted fish was more likely in 2010 than 2012 (95% CI 2.5 to 5.9 times).

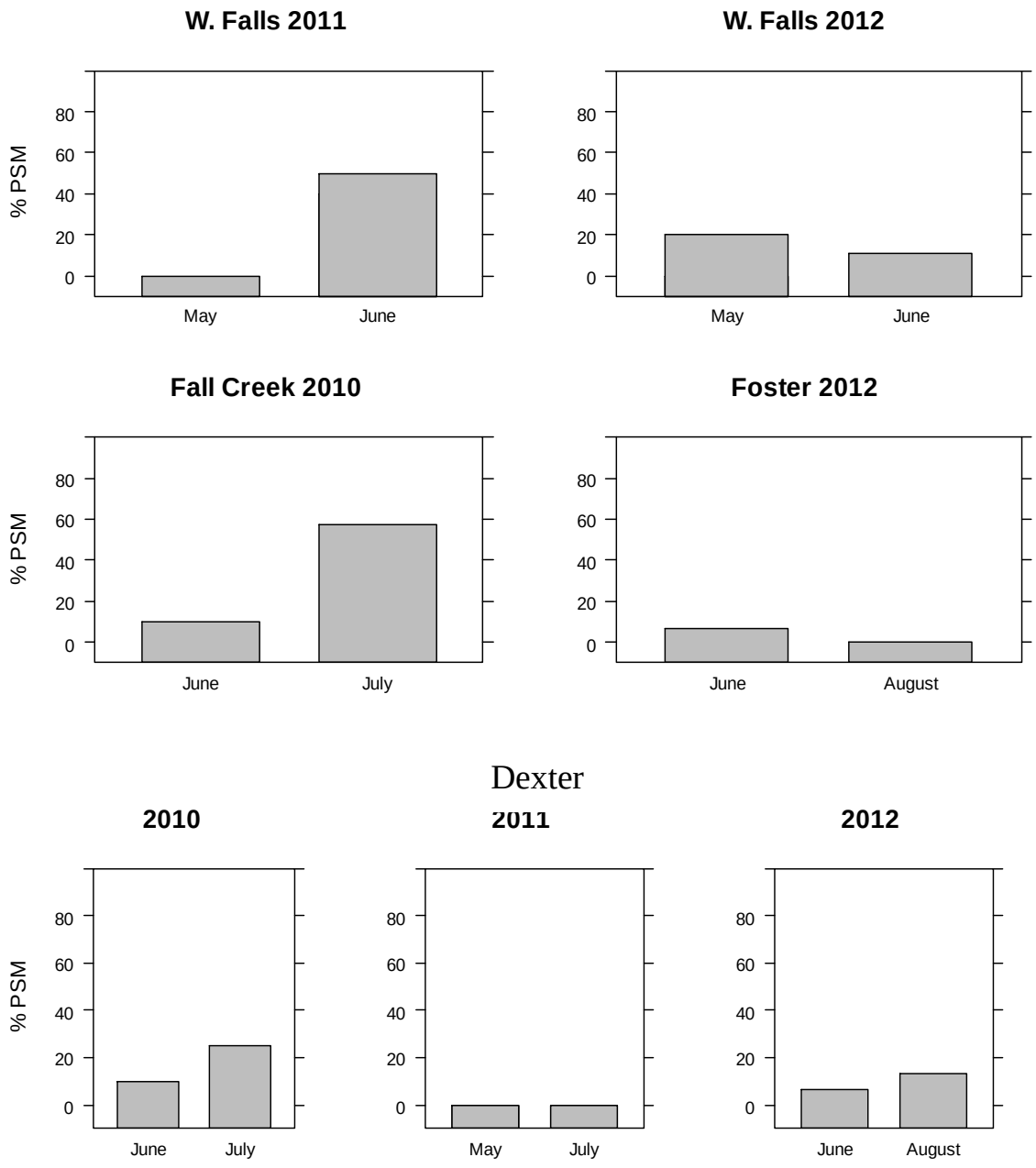

Figure 3. Percent prespawn mortality (PSM) for male and female fish at the holding facility for each collection, location and year. Months indicate collection times for fish transported to the holding facility.

Table 5. Likelihood of PSM for male and female held fish based on multiple logistic regression of collection date, location and year. The odds ratio applies to the first group in the comparison, e.g. PSM is 3.1 times more likely in the late held group versus the early held group (95% CI 0.9 to 12.9), and PSM is 0.2 times less likely in 2012 than 2010 (95% CI 0.0 to 0.8). Statistical significance indicated as follows: p< 0.001 '***'; 0.01'**'; 0.05'*'; 0.1'.'

Comparison	Odds ratio (95% CI)	
Late v. Early	3.1 (0.9-12.9)	.
Dexter v. W. Falls	0.1 (0.0-0.35)	**
Fall Creek v. W. Falls	0.2 (0.0-1.3)	.
Foster v. W. Falls	0.1 (0.0-0.6)	*
2011 v. 2010	0.2 (0.0-1.1)	.
2012 v. 2010	0.2 (0.0-0.8)	*
Female v. Male	0.9 (0.4-2.5)	

Percent PSM at the holding facility is presented in figure 3. There was no mortality directly associated with the capture or transport process. In table 5 all fish from Willamette Falls were coded as an early collection date since collections at Willamette Falls were 'early' relative to upstream collection dates. While this has not been tested for Chinook salmon of the Willamette River, we did not assume that early fish at Willamette Falls were early fish at upstream traps. For Chinook salmon of the Yukon River Eiler (2013) did not find a correlation between entry time and arrival at spawning tributaries. A fish from a late collection date is 3.06 times more likely to experience PSM than a fish from an early collection date (95% CI 0.89 to 12.90 times more likely). Based on the model, a Willamette Falls fish is 14.29 times more likely to experience PSM than a fish from Dexter (95% CI 2.86 to 100 times); we point out here that the sources of mortality between the two groups are likely different, as will be discussed subsequently. There were no differences in the likelihood of PSM between the upper Willamette locations. A fish in the year 2010 was more likely to experience PSM than 2012 (table 5).

Held fish produced viable offspring, regardless of location or collection time.

Pathogens: statistical analysis

A binomial logistic regression model was used to compare all (both held and outplanted) PSM and spawned fish(Table 6). The general model used was fish type (PSM, spawn, live)~ P1+P2…+P7+location+year+collection date, where P1 to P7 indicate the presence/absence of *P. minibicornis, R. salmoninarum, C. shasta*, *N. salmincola*, *Myxobolus sp.*, *A. salmonicida*, and

Apophallus sp. /E. milvi. Location included Fall Creek, Foster and Dexter. Data were used from years 2010 to 2012 and arrival indicates whether collection was "early", "late" or from the river (outplanted fish); this comparison did not include fish from Willamette Falls.

For comparisons involving live fish (i.e. fish sampled directly at trapping facility), multinomial logistic regression models were used. This allowed us to compare live to PSM held (PSM^H) and PSM outplant (PSM^O) (Table 7) or live to spawned held ($spawn^H$) and spawned outplant ($spawn^O$) (Table 8). *A. salmonicida* was excluded; there was complete separation in the model as *A. salmonicida* was not detected in the live group. Further, arrival was not characterized for live comparisons because outplants could not be categorized as either early or late, and live fish were not grouped as either outplants or held (leading to complete separation in the model). Other than these changes, the general model is the same as the one described above for PSM and spawned comparisons.

Table 6. Odds ratios from binomial logistic regression model comparing spawned v. PSM[¥] (for both outplant and held fish) as a function of pathogens, location, year and collection time. *Parvicapsula minibicornis, Renibacterium salmoninarum, Ceratomyxa shasta, Nanophyetus salmincola, Aeromonas salmonicida, Myxobolus sp., Apophallus sp. and Echinochasmus milvi*. Statistical significance indicated as follows: p< 0.001 '***'; 0.01'**'; 0.05'*'; 0.1'.'

Pathogen	Odds ratio (95% CI)		
P. minibicornis +	1.2	(0.4-3.9)	.
R. salmoninarum +	0.2	(0.0-0.7)	***
C. shasta +	7.0	(2.6-21.6)	**
N. salmincola +	0.9	(0.2-4.5)	
A. salmonicida+	1.4	(0.4-4.8)	
Myxobolus sp. +	2.0	(0.7-6.4)	
Apophallus sp./E. milvi +	0.8	(0.3-2.3)	
Foster v. Fall Creek	1.8	(0.1-32.9)	
Dexter v. Fall Creek	0.5	(0.1-1.6)	
2011 v. 2010	0.5	(0.1-2.2)	
2012 v.2010	1.2	(0.1-27.9)	
Early v. Outplant	159.1	(26.2-1747.2)	***
Late v. Outplant	24.4	(7.2-105.7)	***

[¥]This would be read as: A spawned fish was 7 times more likely to have *C. shasta* than a PSM fish (95% CI 2.6-21.6); Spawned fish are 1.8 times more likely than PSM fish at Foster than Fall Creek (95% CI 0.1 to 32.9); Spawned fish are 1.2 times more likely than PSM fish in 2012 than 2010 (95% CI 0.1 to 27.9).

Table 7. Multinomial logistic regression of PSM^H, PSM^O and live fish as a function of pathogens, location and year. Odds ratio and (95% CI) are listed for each comparison[¥]. *Parvicapsula minibicornis, Renibacterium salmoninarum, Ceratomyxa shasta, Nanophyetus salmincola, Aeromonas salmonicida, Myxobolus sp., Apophallus sp. and Echinochasmus milvi*. Statistical significance indicated as follows: p< 0.001 '***'; 0.01'**'; 0.05'*'; 0.1'.'

	PSM^H v. Live			PSM^O v. Live			PSM^H v. PSM^O		
P. minibicornis +	0.2	(0.0-1.8)		0.5	(0.1-1.6)		0.5	(0.1-3.5)	
R. salmoninarum +	36.1	(4.8-268.8)	***	1.1	(0.2-6.4)		32.1	(5.0-205.3)	***
C. shasta +	0.5	(0.1-4.1)		0.7	(0.3-2.0)		0.7	(0.1-6.1)	
N. salmincola +	2.3	(0.1-51.6)		1.3	(0.2-7.3)		1.8	(0.1-30.4)	
Myxobolus sp. +	0.7	(0.1-6.2)		0.3	(0.1-0.9)	*	2.1	(0.2-20.3)	
Apophallus sp./E. milvi +	0.2	(0.0-1.3)		0.3	(0.1-1.0)	.	0.6	(0.1-3.8)	
Foster v. Fall Creek	6.8E+6	(1.5E+6 -3.1E+7)	***	5.6	(0.3-108.9)		9.7E+5	(2.0E+5-4.7E+6)	***
Dexter v. Fall Creek	1.9	(0.2-16.0)		2.9	(0.7-11.7)		0.7	(0.1-4.7)	
2011 v. 2010	0.2	(0.0-4.7)		0.4	(0.1-1.9)		0.5	(0.0-9.7)	
2012 v. 2010	4.8E-8	(1.1E-8 -2.2E-7)	***	0.0	(0.0-0.5)	*	1.3E-6	(2.8E-7 -6.5E-6)	***

[¥] This would be read as: PSM^H fish are 36.1 times more likely to have *R. salmoninarum* than live fish (95% CI 4.8 to 268.8), PSM^H fish are 1.9 times more likely than live fish at Dexter compared to Fall Creek(95% CI 0.2 to 16.0) and PSM^H fish are 0.2 times less likely than live fish to occur in 2011 than 2010 (95% CI 0.0 to 4.7).

Table 8. Multinomial logistic regression of Spawn^{H}, Spawn^{O} and live fish as a function of pathogens, location and year. Odds ratio and (95% CI) are listed for each comparison[¥]. *Parvicapsula minibicornis, Renibacterium salmoninarum, Ceratomyxa shasta, Nanophyetus salmincola, Aeromonas salmonicida, Myxobolus sp., Apophallus sp. and Echinochasmus milvi*. Statistical significance indicated as follows: p< 0.001 '***'; 0.01'**'; 0.05'*'; 0.1'.'

	Spawn^{H} v. Live			Spawn^{O} v. Live			Spawn^{H} v. Spawn^{O}		
P. minibicornis +	0.3	(0.1-0.9)	*	1.5	(0.4-6.0)		0.2	(0.1-0.8)	*
R. salmoninarum +	3.6	(1.1-12.5)	*	0.7	(0.1-8.0)		5.1	(0.6-46.8)	
C. shasta +	4.1	(1.9-8.8)	***	5.8	(1.7-19.7)	**	0.7	(0.2-2.3)	
N. salmincola +	0.6	(0.1-2.3)		0.9	(0.1-7.4)		0.6	(0.1-4.0)	
Myxobolus sp. +	0.8	(0.4-1.7)		0.1	(0.0-0.5)	**	5.6	(1.6-20.6)	**
Apophallus sp./E. milvi +	0.5	(0.2-1.1)	.	0.2	(0.0-0.6)	**	3.3	(0.9-11.7)	.
Foster v. Fall Creek	1.6	(0.3-9.0)		4.8E+6	(2.3E+6- 1.0E+7)	***	4.1E-7	(1.4E-7- 1.2E-6)	***
Dexter v. Fall Creek	2.4	(0.7-8.7)		0.8	(0.2-3.9)		3.0	(0.7-12.6)	
2011 v. 2010	1.1	(0.3-3.8)		0.1	(0.0-0.6)	*	14.0	(2.1-93.9)	**
2012 v. 2010	1.5	(0.4-5.9)		6.4E-8	(3.0E-8- 1.4E-7)	***	2.0E+7	(7.6E+6- 5.1E+7)	***

[¥] This would be read as: Spawn^{H} fish are 3.6 times more likely to have *R. salmoninarum* than live fish (95% CI 1.1 to 12.5), spawn^{H} fish are 2.4 times more likely than live fish at Dexter compared to Fall Creek(95% CI 0.7 to 8.7) and spawn^{H} fish are 1.1 times more likely than live fish to occur in 2011 than 2010 (95% CI 0.3 to 3.8).

For all fish (held and outplant), PSM were 5.9 times more likely to have *R. salmoninarum* than spawned fish (Table 6; 95% CI 1.5 to 25.0). Spawned fish are 7.0 times more likely to have *C. shasta* than PSM (2.6 to 21.6). There was no difference between spawned fish and PSM for the remaining pathogens.

PSM^{H} were 36 times more likely to be positive for *R. salmoninarum* than live fish (Table 7; 95% CI 4.84 to 268 times), after accounting for year and location. Live fish were 3.3 times more likely to be positive for *Myxobolus sp.* than PSM^{O} (95% CI 1.1 to 10). Finally, PSM^{H} were 32 times more likely to be *R. salmoninarum* positive than PSM^{O} (95% CI 5.0 to 205.3).

$Spawned^{H}$ fish were3.6 and 4.1 times more likely to be positive for *R. salmoninarum* and *C. shasta* than live fish (Table 8; 95% CI 1.1 to 12.5 and 1.9 to 8.8, respectively), after accounting for year and location. Conversely, live fish were 3.23 times more likely to be positive for *P. minibicornis* than $spawned^{H}$ fish (95% CI 1.16 to 9.09).

$Spawned^{O}$ fish were 5.8 times more likely to have *C. shasta* than live fish (Table 8; 95% CI 1.7 to 19.7). However, live fish were 7.1 and 6.3 times more likely to have *Myxobolus sp.* and *Apophallus sp./E. milvi* (95% CI 1.9 to 25.0 and 1.6 to 25.0 respectively).

$Spawned^{H}$ fish were 5.6 times more likely to have *Myxobolus sp*. than $spawned^{O}$ fish (Table 8; 95% CI 1.6 to 20.6). Conversely, $spawned^{O}$ fish were 4.8 times more likely to have *P. minibicornis* than $spawned^{H}$ fish (95% CI 1.3 to 16.7).

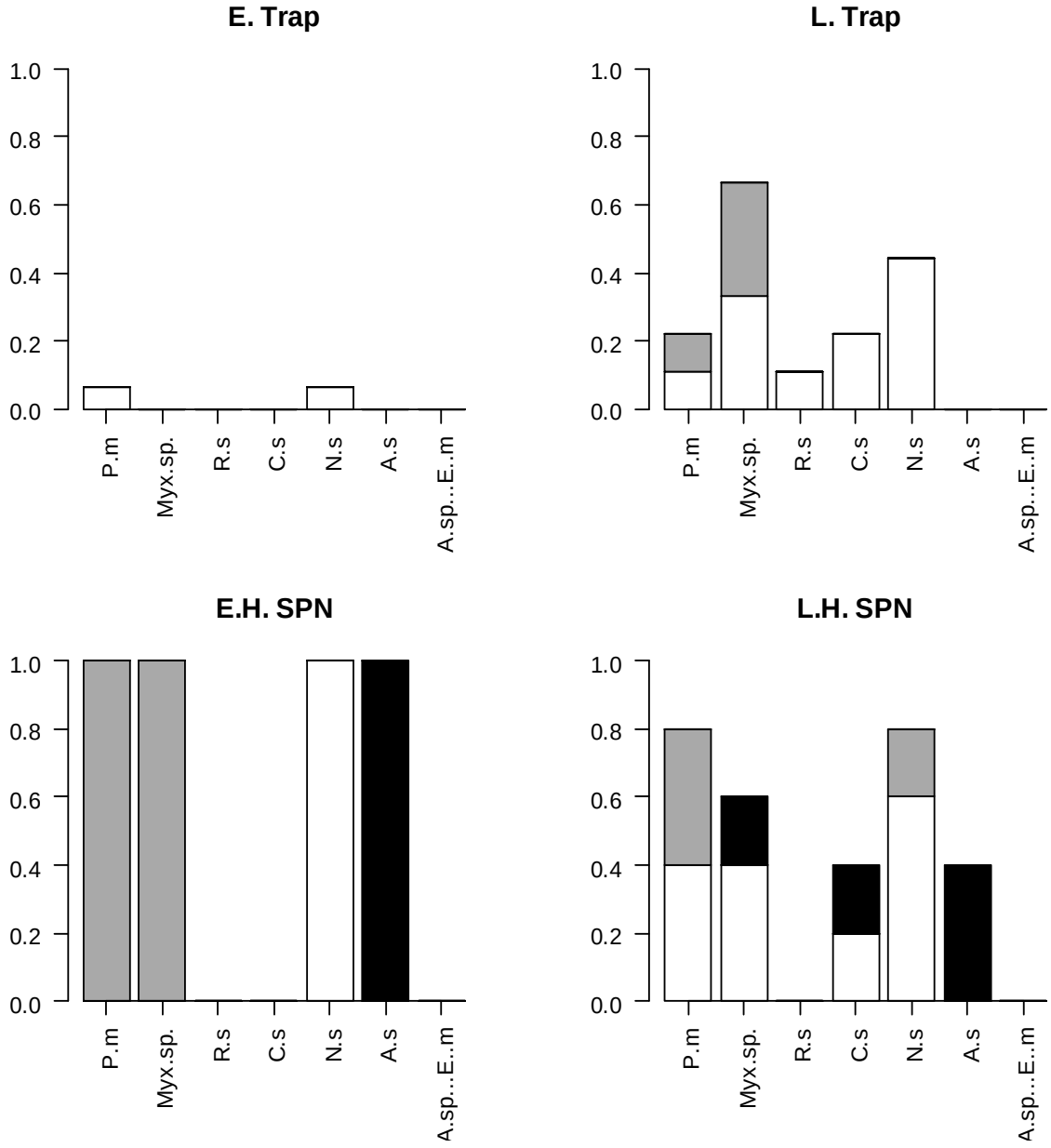

Figure 4. Pathogen prevalence and severity for fish sampled at Willamette Falls early (E. Trap) and late (L.Trap) and held spawned fish collected from Willamette Falls early(E.H. SPN) and late (L.H. SPN) in 2011. P.m=*Parvicapsula minibicornis*; Myx. sp.= *Myxobolus sp*; R.s=*Renibacterium salmoninarum*; C.s= *Ceratomyxa shasta*; N.s= *Nanophyetus salmincola*; A. s= *Aeromonas salmonicida*; A.sp..E.m=*Apophallus sp.* and *Echinochasmus milvi.*

Fish collected from Willamette Falls were not included in the models above for two reasons. First, we did not have complete groups to compare, e.g. there is no outplant group to compare with early and late held fish. Secondly, there was complete separation of data for most pathogens

when comparing held fish and fish sampled at the falls (Figure 4). In other words, while fish sampled at Willamette Falls show low prevalence of infection with pathogens, these infections develop with time (i.e. pathogens are more prevalent and severe in spawned fish). For this location and others, we graphed the prevalence and severity of each pathogen by year, location and collection type (Appendix A).

Discussion

The primary focus of this study was to determine if salmon held in a pathogen free, constant temperature environment have lower PSM than those in the river (H1). We found that both early and late held female fish are less likely to experience PSM than an outplanted female fish, after accounting for location and year. Our study design did not allow us to test this eventuality for males, but holding males would likely decrease PSM compared to outplanted males because sex did not significantly affect the likelihood of PSM in held fish. We also found that early held fish are less likely to experience PSM than late held fish (H2). The differences between early held, late held and outplanted fish could be that less exposure to river conditions is beneficial to reducing PSM. However other factors could attribute to this difference, including differences in fish condition, stock, trap and transport operations, temperature, or pathogen acquisition, as well as other less obvious fish, environmental, or management factors.

Estimates of PSM for outplanted fish are based on carcasses recovered during river surveys over the total number of fish transported. It is important to note that there is some degree of error associated with sampling efforts. Additionally, this estimate of PSM only includes fish that survive to upstream trapping facilities. If we were to account for the mortality that occurs prior to reaching upstream sites, it is likely that the estimated PSM would be higher. While our current estimate of PSM is conservative and reduces the scope of inference to those fish that survive to upstream trapping facilities, it is still useful in determining the efficacy of holding prior to outplanting to reduce PSM (H1).

As percent PSM varies by year, the effectiveness of holding does as well—i.e. in years where percent PSM is higher in outplants there is comparatively less PSM at the holding facility, but when percent PSM is low in outplants, it is also low at the holding facility. As a management tactic, holding would be most effective in years where high PSM is expected and less helpful in

low PSM years. Currently a model by Dr. Michael Colvin, Oregon State University and others incorporates temperature, flow, migration timing and other parameters with the goal of determining the best transporting and holding practices that balances fish survival and financial cost (Schreck et al. 2013). If we could predict good versus bad PSM years, one could hold fish as needed.

While our study cannot directly attribute lower PSM at the holding facility to the cool constant temperatures and a parasite-free environment, those are the most plausible explanation for the results. Other studies provide evidence of the correlation between elevated temperatures and PSM (Crossin et al. 2008; Keefer et al. 2010). Jeffries et al. (2012) found an upregulation in genes involved in immunity in sockeye held at 19 °C. They suggest that while this could be due to temperature stress, it could also be a response to higher virulence of pathogens that progress in temperature dependent manner.

With the exception of one fish, PSM fish at our holding facility exhibited a high prevalence and severity of *R. salmoninarum* or *A. salmonicida.* We speculate that the stresses experienced by the fish prior to our collection and/or the collection and transportation process itself may have resulted in proliferation of the pathogens. This is suggested when we examine percent prevalence and severity of bacterial infections of fish sampled at the trapping facility, holding facility, and outplant locations (Appendix A, figures 5-6). Fish at the trapping facility have little or no histological evidence of *R. salmoninarum* or *A. salmonicida* infections, and prevalence remained low in outplanted fish. Meanwhile, PSM at the holding facility show a high prevalence of infection with these pathogens.

That we see a higher prevalence of bacterial infection at the holding facility is not surprising. *R. salmoninarum* has long been a major problem for cultured salmon (Fryer and Sanders 1981) and the infection is very prevalent in wild Chinook salmon in the Pacific Northwest (Banner et al. 1986; Pascho and Murray 1987; Arkoosh et al. 2004; Rhodes et al. 2006). *R. salmoninarum* is a slow growing bacterium; it takes weeks before becoming fatal and horizontal transmission is similarly slow (Murray et al. 1992; McKibben and Pascho 1999). Therefore, many of the fish

that are collected as adults from the Willamette River have subclinical infections that may become clinical several weeks after capture or being subjected to other stressors.

With *A. salmonicida*, one highly infective fish can spread it to other fish. The density dependent transmission of *A. salmonicida* makes it an especially important consideration for holding facilities, where fish would presumably be held in close quarters (Ogut & Reno, 2004). The Willamette Falls fish held in 2011 are the best example of this, where 100 percent of PSM fish were moderately or severely infected with *A. salmonicida* (Appendix A, Figure 3). These fish died within a month of transport, most within the first two weeks after transport. Not only does this emphasize the importance of this disease, it calls attention to the importance of reducing transport and handling stress. Bacterial infection could also explain why held fish from Willamette Falls were more likely to experience PSM than upstream fish (table 5). It is possible that fish experience pathogen associated mortality between Willamette Falls and the trapping facilities. This would bias the data making it look like PSM was less upstream when in fact it could have been more.

Both *R. salmoninarum* and *A. salmonicid*a are common to hatcheries, which usually treat for bacterial infections with antibiotics. The effectiveness of antibiotics can be seen in Appendix A, Figures 5-7 —*A. salmonicida* was not detected histologically in spawned fish from Willamette Hatchery, while we found residual levels of infection in held spawned fish. Infection with *R. salmoninarum* was equal to or less than that of spawned fish at the holding facility, and severity of infection was generally lower at Willamette Hatchery.

Our held fish were not treated with antibiotics in an effort to keep them similar to outplanted fish. If holding were used as a management tactic, fish would be held for a period of time and then released to spawn. Based on the prevalence of bacterial disease in held fish, careful consideration will have to be given to the use of antibiotics. We know that fish in a held setting can experience pathogen associated mortality from these diseases. There is also a possibility that stress (e.g., handling and transport to release site) and lack of treatment could increase the chances of death before spawning. Additionally, the ODFW fish health management policy

("Fish Health Management" 2003) is designed to prevent the holding and transportation of fish that will either incubate or release high levels of bacteria into environment.

While antibiotics would help to alleviate the issues in the previous paragraph, this approach has certain concerns. The presence of antibiotics in a fish may select for drug resistant bacteria or bacteria with reduced susceptibility to antibiotic (Bell et al. 1988; Rhodes et al. 2008). In addition, antibiotics that are frequently used to treat fish also are effective for pathogens of other animals, hence there is the potential that using them for fish could create an environment for drug resistant bacteria that affect terrestrial livestock and even humans (Kemper 2008). A previous study by Keefer et al. (2010) examined the effect of antibiotic treatment on survival of spring Chinook salmon, but the results were inconclusive. Ideally, there would be a balance between release of bacteria and/or antibiotic and survival of held fish.

The second aim of this study was to examine the suite of pathogens infecting adult salmon to determine whether PSM fish and spawned fish had similar pathogen burdens (H3) and whether held fish had lower pathogen burdens than outplanted fish (H4). While held PSM died from bacterial infections, no single pathogen stood out as the culprit of PSM in outplanted fish. It is interesting to note that in outplanted fish, a PSM fish had a severity of *N. salmincola* comparable to that of a spawned fish. *P. minibicornis* was another parasite that appeared in PSM fish at levels comparable to a spawned fish, both in the river and at the holding facility.

A PSM fish with pathogen burdens similar to that of a spawned fish could be explained several ways. First, it is possible that a PSM fish is more susceptible than a successful spawner to infection with this parasite. What would cause this increased susceptibility (e.g. fish stock, year class, ocean conditions, previous life history, stress, pathogen burden, a weakened immune system, senescence, temperature) cannot be determined from this study. Other research with sockeye salmon have found decreased osmoregulatory function to be indicative of mortality (Jeffries et al. 2011). Both *N. salmincola* and *P. minibicornis* infect the kidney and gills, key areas of osmosregulatory function, and higher levels of infection could perhaps indicate lowered osmoregulatory ability.

A similar pathogen burden between PSM and spawned fish could also indicate that fish experiencing PSM are spending more time in freshwater. Spending more time in freshwater could affect timing of senescence, the amount of pathogen exposure and burden, the degree days acquired, the amount of time holding below trapping facilities (dams) and on spawning grounds, the amount of energy reserves applied to migration, and more.

When we compared all PSM fish to all spawned fish, we found PSM fish were more likely to have *R. salmoninarum* and spawned fish were more likely to have *C. shasta* (table 6). The majority of PSM at the holding facility was a result of *R. salmoninarum*. However, it is likely that all fish (live, PSM, or spawned fish at both the holding facility or outplanted) are infected with this pathogen and subclinical infections are hard to detect with histology (Kent et al. 2013). The polychaete alternate host of *C. shasta*, and hence the infectious stage of the parasite, occurs in lower reaches of rivers (Hallet and Bartholomew 2012). *C. shasta* is a proliferative pathogen that has a degree day dependent life cycle and infections are initiated after water temperatures rise in the late spring or early summer (Johnson 1975; Chiaramonte 2013). Because spawned fish spend the most time in the river (fish spawn in September, and live and PSM fish were collected May through August), it follows that we would be more likely to detect this pathogen in spawned fish than either live or PSM fish.

Spawned^{H} fish were more likely to have *Myxobolus* sp. and less likely to have *P. minibicornis* compared to spawned^{O} fish. *P. minibicornis* also uses the freshwater polychaete *Manayunkia speciosa* as an alternate host (Bartholomew et al. 2006), and thus it is likely that salmon do not become infected until they return to freshwater to spawn. In this case, removing adult salmon from the river early in the summer may reduce the prevalence of infection. Salmon are infected with *Myxobolus* sp. as juveniles in freshwater and infections persist to adulthood (Kent et al. 1993; Kent et al. 1994; Ferguson et al. 2008). The lower likelihood of *Myxobolus* sp. in spawned^{O} fish than in either live or spawned^{H} fish suggests that outplanted fish with this infection are dropping out of the population (and holding may help survival). The infection targets the hind brain and spinal cord; Moles and Heifetz (1998) reported that sockeye salmon (*Oncorhynchus nerka*) infected with a very similar neural parasite, *Myxobolus arcticus*, have significant reduced swimming speed of compared to uninfected fish. However, live fish were

also more likely to have *Myxobolus* sp. infections than PSM^{O} fish, which does not support the hypothesis that the parasite is associated with PSM. It could be that our samples size is not adequate enough to detect *Myxobolus sp.* in PSM ^{O} fish.

Spawned^{O} fish were less likely to have *Apophallus sp.* and *E. milvi* than live fish, while PSM^{O} were equally likely as live fish to have these pathogens. Both of these pathogens are non proliferative and encyst in the fish host (Ferguson et al. 2011). Therefore, we'd expect spawned fish to be either equally likely or more likely (depending on where in freshwater exposure occurs) to have infections with these pathogens than live fish. Because we see the opposite could indicate fish with these infections are not surviving to spawn and that the certain pathogens are causing PSM. This is supported by the equal likelihood of live and PSM^{O} fish having these pathogens.

The similar prevalence of infection between spawned^{O} and spawned^{H} fish for several of the pathogens in study was not surprising as they were taken from the same population and two groups were likely exposed to the same pathogens before capture. Moreover, there could be differences that I was not able to reveal due to statistical limitations, such as relatively small sample sizes and replicates, and several confounding factors such as time and location in the river and variability in PSM and temperatures between years.

Nevertheless, similar severity of infection could indicate several things: there is some stress associated with holding, fish are senescing and proliferative pathogens multiply regardless of location. It also suggests that interactive effects may be important for mortality (e.g. both pathogens and warm temperatures are needed before significant mortality is evident). Crossin et al. (2008) held fish at ambient and higher temperatures and found more pathogen related mortality at higher temperature. While we use higher temperatures as an example, it could also be an interaction with low flow, holding below trapping facilities, transport stress, etc.

In conclusion, fish held in pathogen free, constant temperature water had lower PSM than fish outplanted, indicating that holding could be a viable method to increase survival to spawn. The majority of the mortality in held PSM fish was due to *R. salmoninarum* and *A. salmonicida*, both

of which are exacerbated by stress and holding. Therefore, careful consideration should be given not only to the transport and handling methods, but also to the use of antibiotics in held fish. Holding as a management tactic would involve the release of held fish into spawning tributaries, which our study did not examine. Future work should hold and release fish, accounting for antibiotics as well as collection and release timing. PSM fish are dying from pathogen associated mortality, based on the similar pathogen burdens of PSM and spawned fish. The next step would be to figure out if this is simply a function of exposure time or if PSM fish more susceptible to infection. Finally, it is important to remember this study mostly examines fish that survive to reach the upper tributaries and thus our scope of inference is limited to these fish. An examination of the prevalence and cause of PSM between Willamette Falls and upstream tributaries would not only broaden our understanding of PSM but potentially identify other management tactics to increase survival to spawn.

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APPENDICES

Appendix A: Pathogen prevalence and severity for held, trapped, and outplanted fish by location and year.

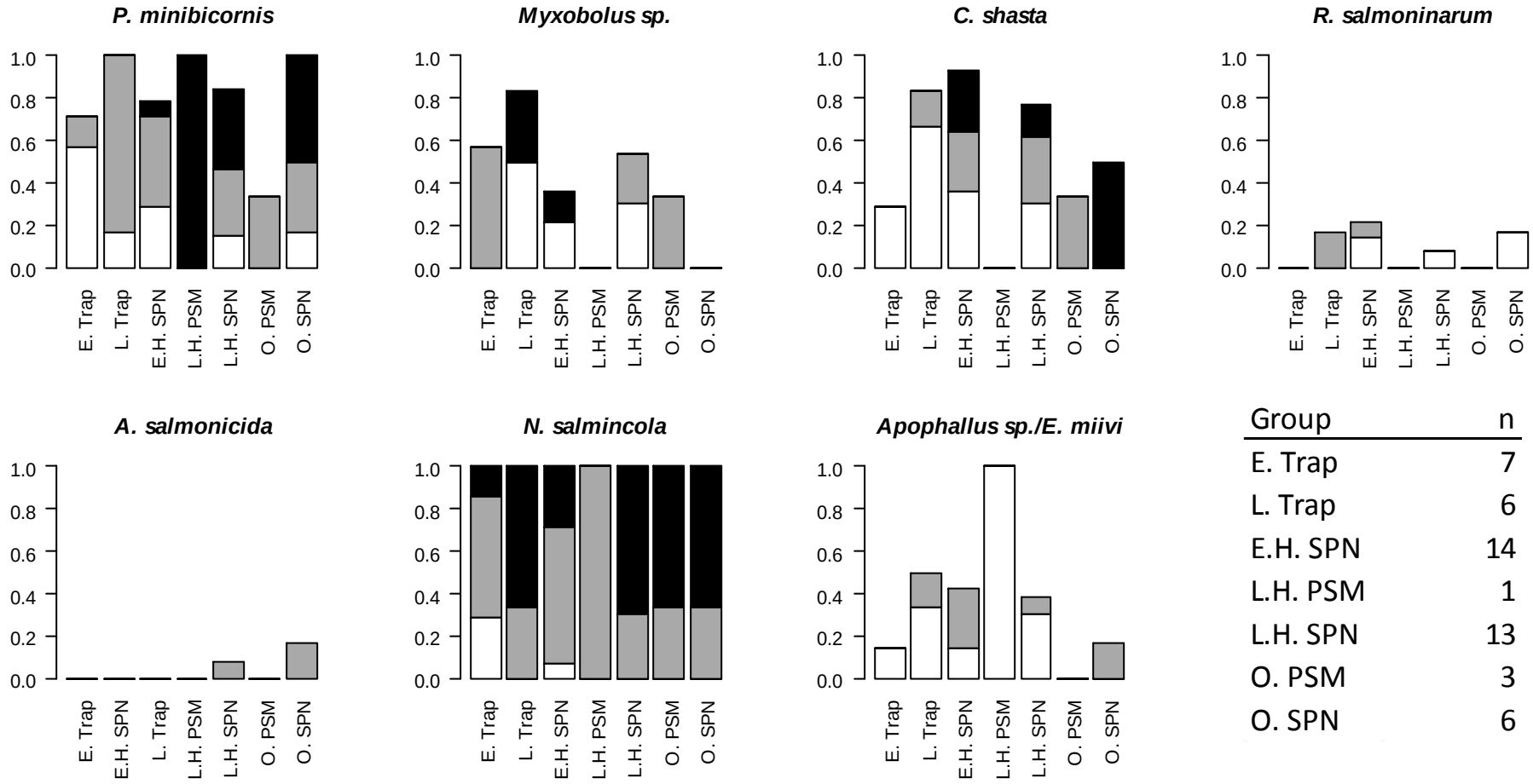


Figure 1. Prevalence of infection in fish obtained from Foster in 2012. Y axis is % prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn); O.PSM (ouplant PSM); O. SPN (outplant spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.

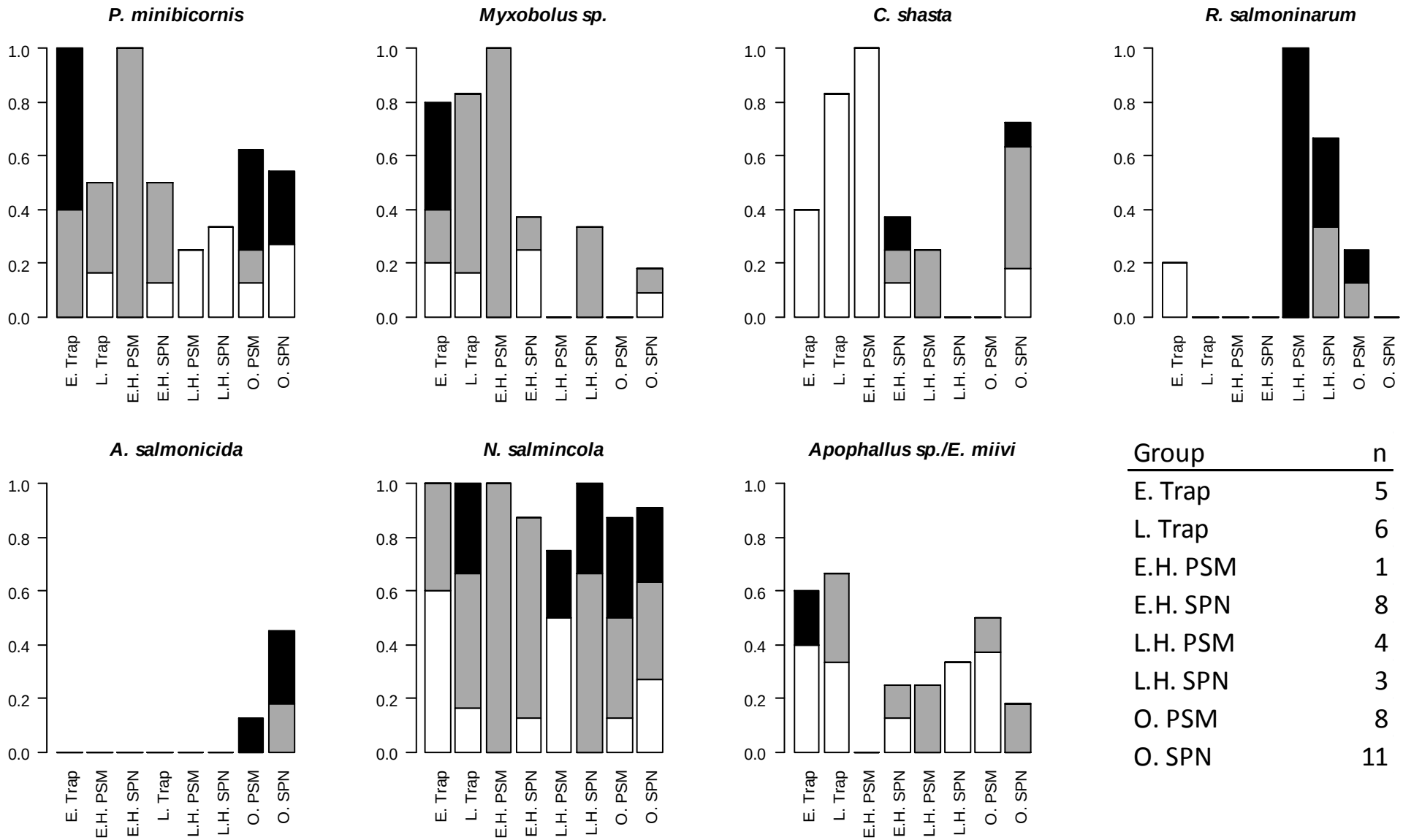


Figure 2. Prevalence of infection in fish obtained from Fall Creek in 2010. Y axis is % prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. PSM(early held PSM); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn); O.PSM (ouplant PSM); O. SPN (outplant spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.

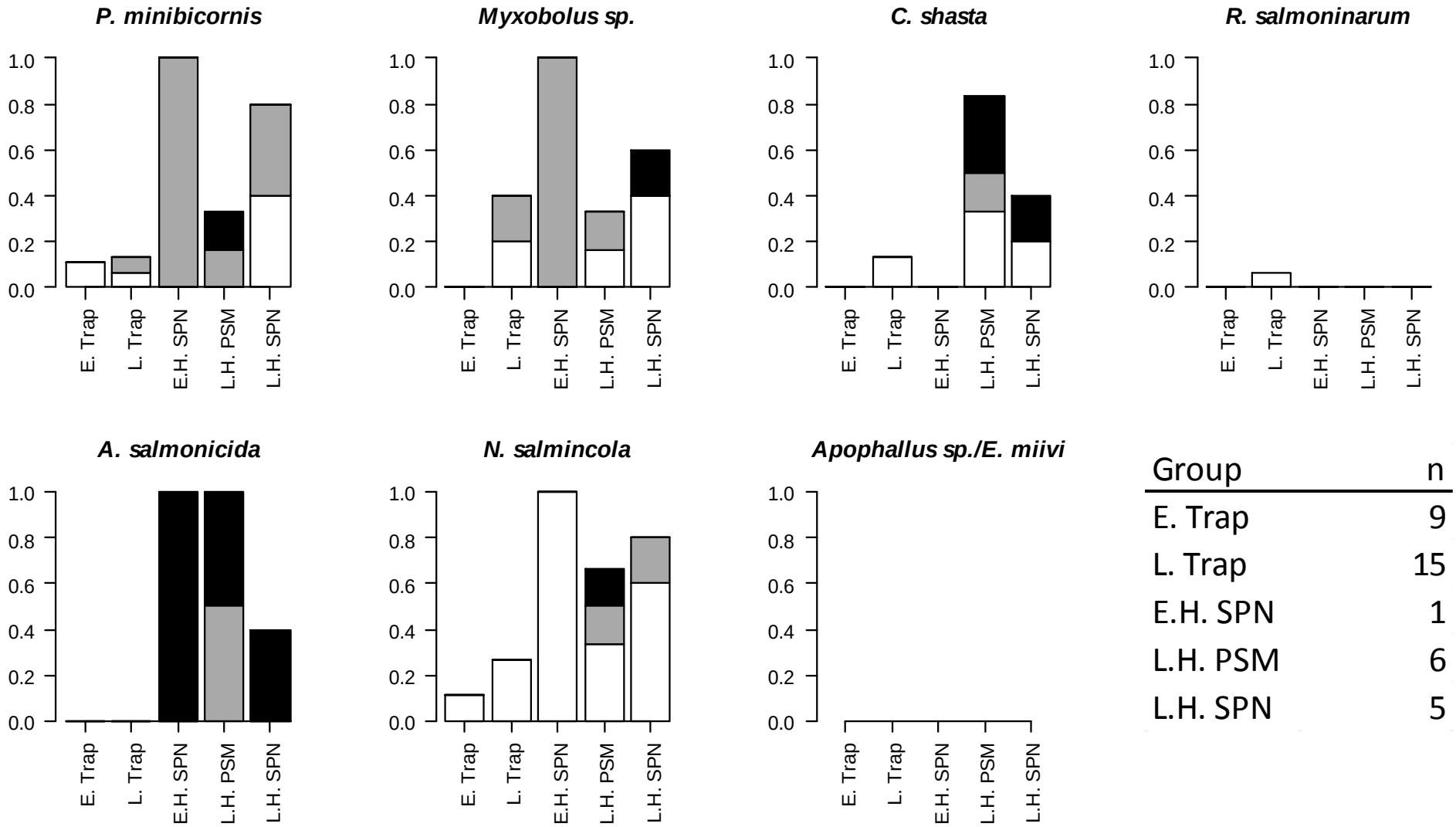
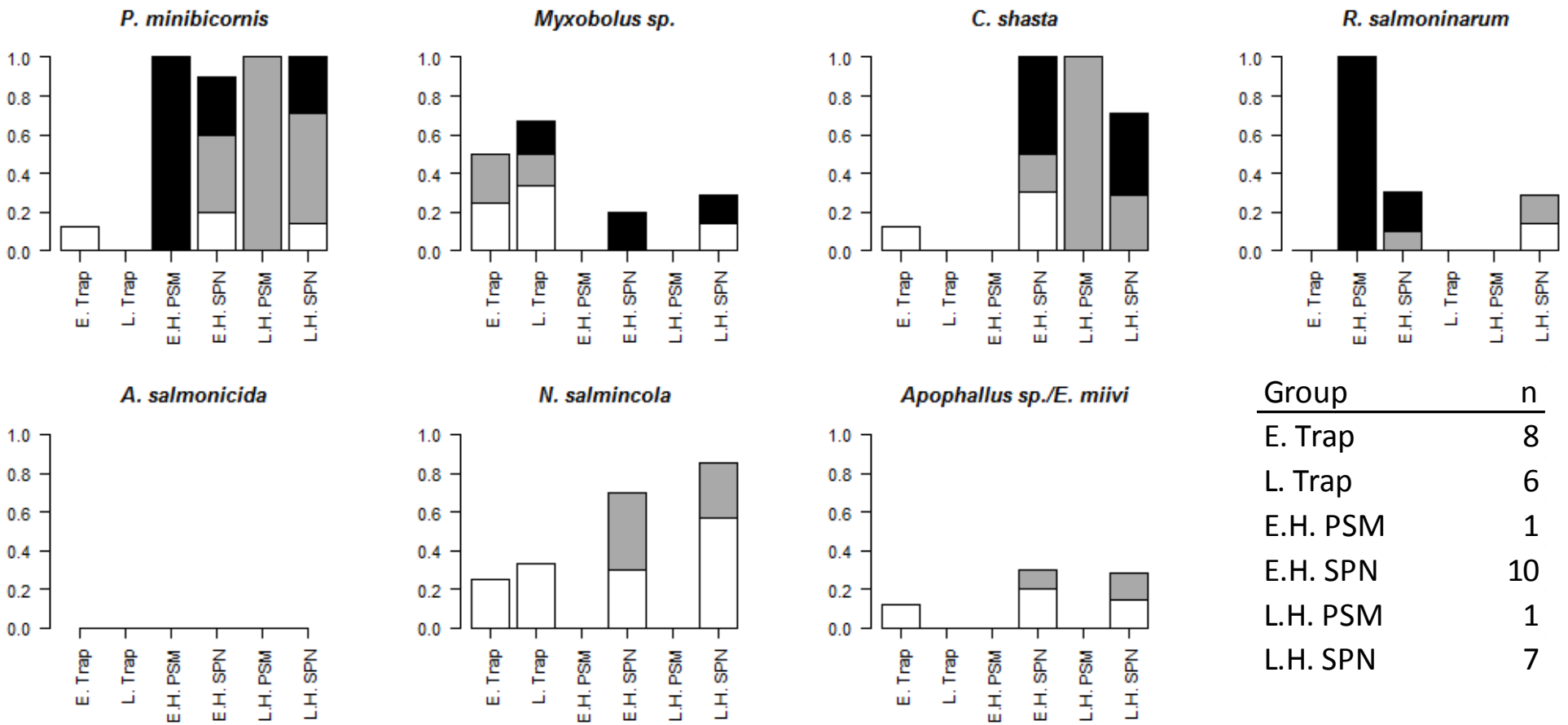
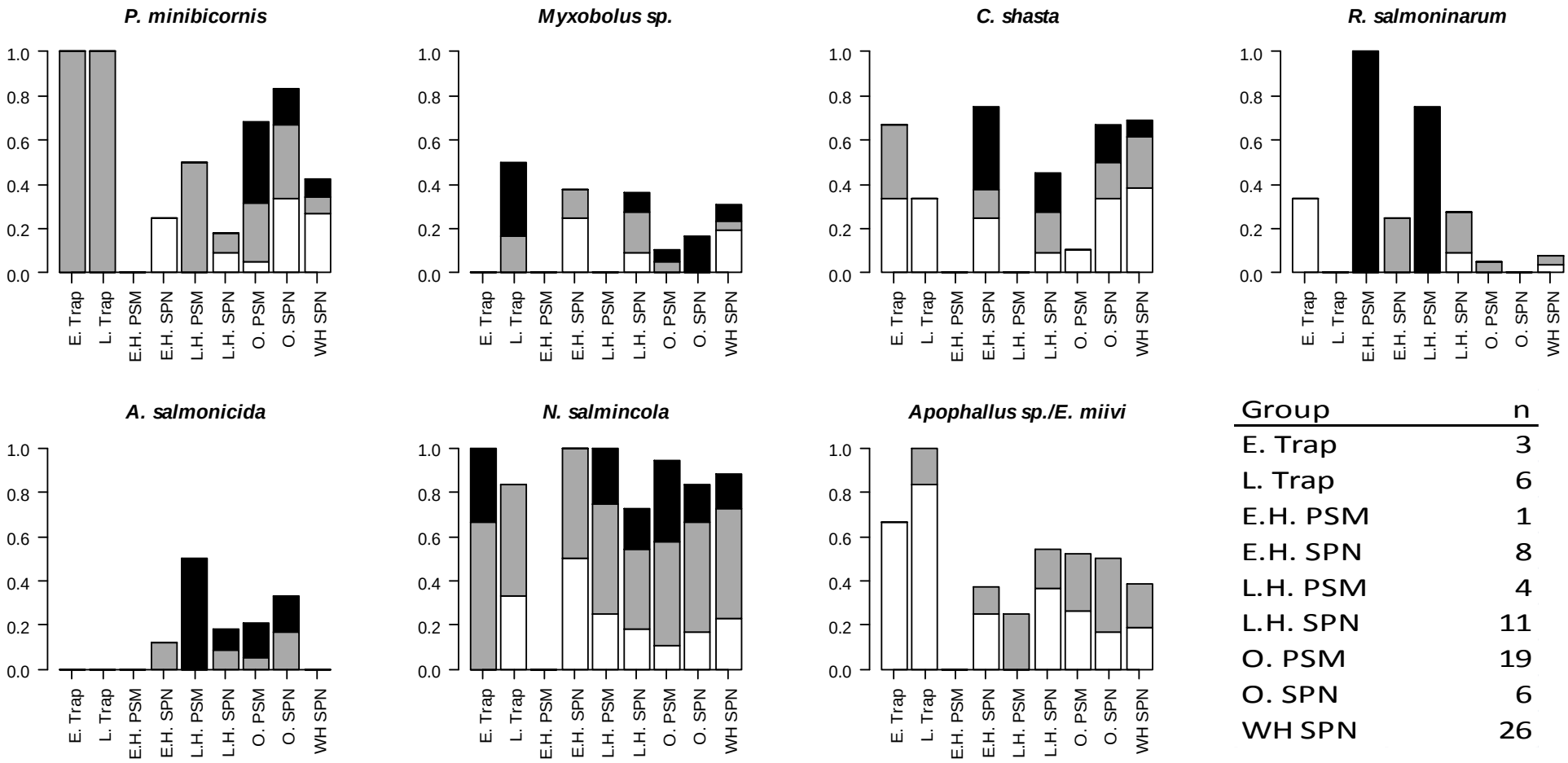


Figure 3. Prevalence of infection in fish obtained from Willamette Falls in 2011. Y axis is % prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. PSM(early held PSM); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.



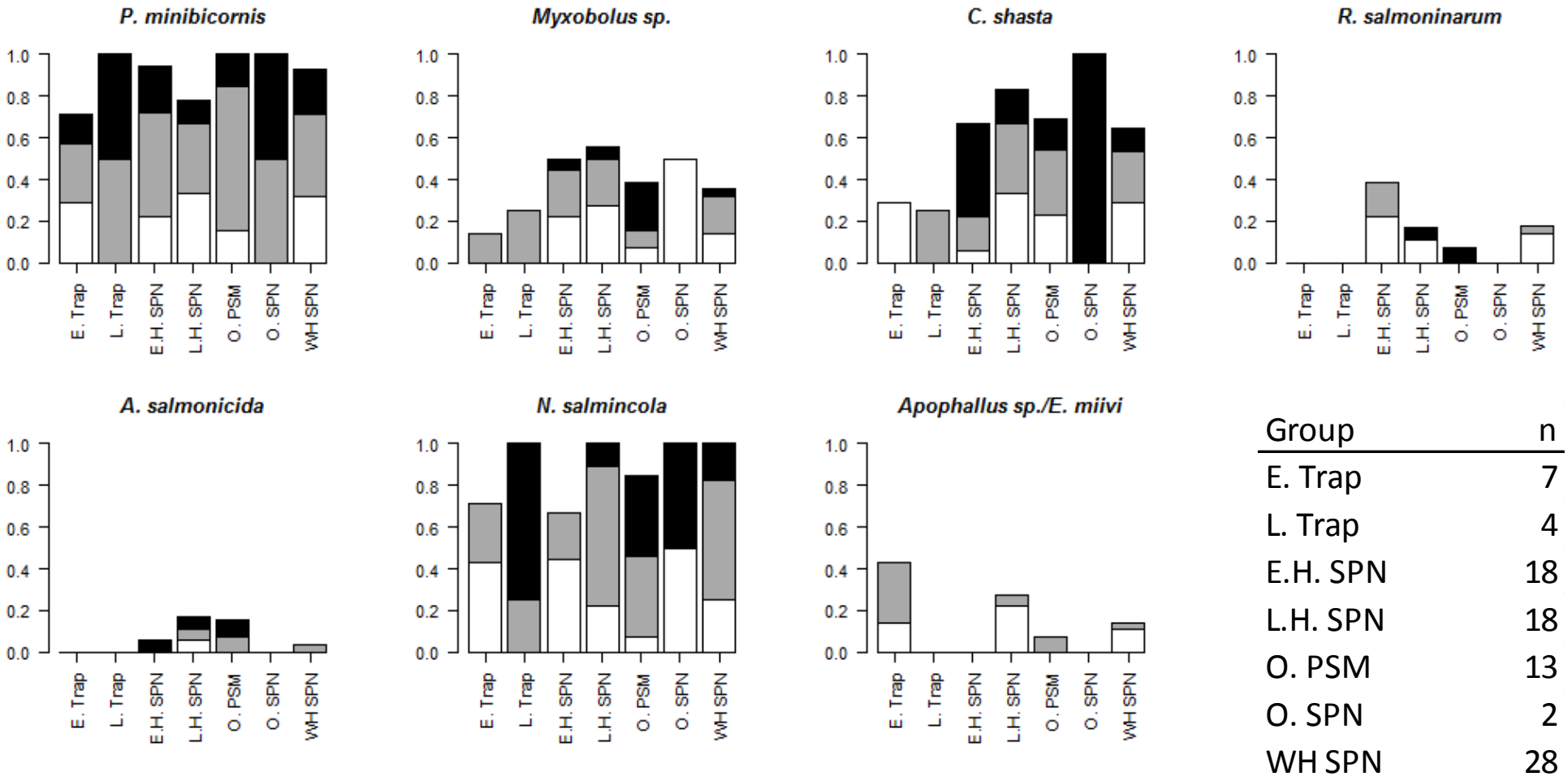
Group	n
E. Trap	8
L. Trap	6
E.H. PSM	1
E.H. SPN	10
L.H. PSM	1
L.H. SPN	7

Figure 4. Prevalence of infection in fish obtained from Willamette Falls in 2012. Y axis is % prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. PSM(early held PSM); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.



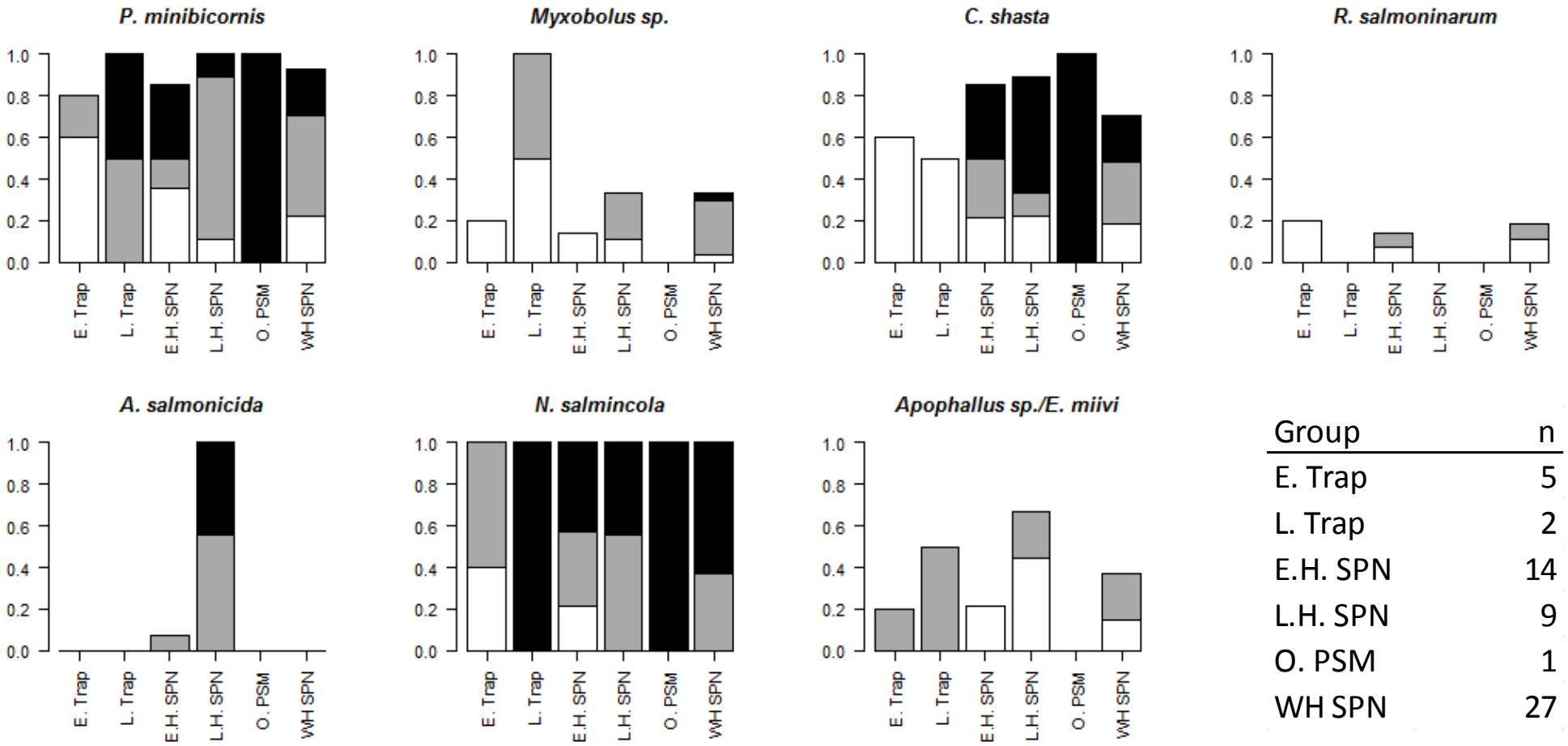
Group	n
E. Trap	3
L. Trap	6
E.H. PSM	1
E.H. SPN	8
L.H. PSM	4
L.H. SPN	11
O. PSM	19
O. SPN	6
WH SPN	26

Figure 5. Prevalence of infection in fish obtained from Dexter in 2010. Y axis is prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. PSM(early held PSM); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn); O.PSM (ouplant PSM); O. SPN (outplant spawn); WH SPN (Willamette Hatchery spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.



Group	n
E. Trap	7
L. Trap	4
E.H. SPN	18
L.H. SPN	18
O. PSM	13
O. SPN	2
WH SPN	28

Figure 6. Prevalence of infection in fish obtained from Dexter in 2011. Y axis is % prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. PSM(early held PSM); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn); O.PSM (ouplant PSM); O. SPN (outplant spawn); WH SPN (Willamette Hatchery spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.



Group	n
E. Trap	5
L. Trap	2
E.H. SPN	14
L.H. SPN	9
O. PSM	1
WH SPN	27

Figure 7. Prevalence of infection in fish obtained from Dexter in 2012. Y axis is % prevalence, x axis indicates fish location and time: E. Trap(early trap); L.Trap(late trap); E.H. PSM(early held PSM); E.H. SPN(early held spawn); L.H. PSM (late held PSM); L.H. SPN (late held spawn); O.PSM (ouplant PSM); O. SPN (outplant spawn); WH SPN (Willamette Hatchery spawn). *Parvicapsula minibicornis, Myxobolus sp., Ceratomyxa shasta, Renibacterium salmoninarum, Aeromonas salmonicida, Nanophyetus salmincola, Apophallus sp. and Echinochasmus milvi*. The colors indicate severity of infection: white = score of 1, light infection; gray= score of 2, moderate infection, black = score of 3, severe infection.

Appendix B: Effect of cortisol on susceptibility of infection with a non-proliferative pathogen.

Introduction

Adult spring Chinook salmon in the Willamette Valley have a range of pathogens including bacteria, myxospores, and trematodes (Kent et al. 2013). Bacteria and myxospores are capable of replicating in the fish host, whereas trematodes are non-proliferative. Of particular interest is a trematode called *Nanophyetus salmincola*, which is found in variable levels in live, prespawn and spawned fish (Schreck et al. 2011). It is possible that the variation is due to time in the river (i.e. more time in river, higher levels of infection), or that immune suppression is involved. If immune suppression is not playing a role, then this parasite could potentially be used to estimate time in freshwater, (after accounting for location, temperature, other hosts, etc). With this in mind, we hope to determine if elevated cortisol levels makes the host more susceptible to a non-replicating pathogen.

Stress is associated with lowered immune competence (Schreck 1996; Schreck et al. 2001); therefore, a stressed host could be more susceptible to pathogens. An example of stress acting as an immune suppressant would be sub-lethal copper exposure and subsequent disease in rainbow trout (Schreck & Lorz 1978; Hetrick et al. 1979). Furthermore, stressed hosts are more susceptible to a higher infection with proliferative pathogens (Lacoste et al. 2001). Indeed, cortisol is actually a growth factor for certain pathogens (Freestone et al. 2008)!

While there is evidence for stress (or high levels of cortisol) causing increased susceptibility to infection with proliferative pathogens (Maule et al. 1987; Vanderkoi et al. 2001), there are relatively few studies that investigate the relationship with non- proliferative pathogens. Of two prominent examples, Kiesecker 2002 and Markkula et al. 2007, only the latter involves a fish host. In both cases, stress made the host immune suppressed, and therefore more likely to have higher burdens of non-proliferative pathogens.

Nanophyetus salmincola was chosen as a model species of parasite due to its presence in spring Chinook salmon, the ability to experimentally infect with this parasite, and its lack of proliferation in salmonid host. Additionally, *N. salmincola* has physiological effects on the

salmonid host, including immune suppression and decreased growth (Jacobson et al. 2003; Ferguson 2011 respectively).

Methods

Fish and Diet:

Spring Chinook salmon from Oregon Department of Fish and Wildlife Marion Forks Hatchery (2010 brood year) were held at the Fish Performance and Genetics Laboratory (FPGL) and fed to satiation with commercial salmon diet. Prior to the start of the experiment, fish fasted for three days to reduce stress that can happen during handling of fed fish (Ramsay et al. 2006). Once transferred to the Salmon Disease Laboratory (SDL), fish were again fed to satiation daily with commercial salmon diet.

Cortisol injections and Fin clips:

Cortisol was dissolved into molten cocoa butter (40 ˚C) at a ratio of 4 mg cortisol/ 1ml cocoa butter. Then, as the cocoa butter cooled (around 30 ˚C) but was not yet solidified, 100 ul was intra-peritoneally injected into the anesthetized fish. Fish were anesthetized with 50 mg/L tricaine methanesulfonate (MS 222) buffered to pH of 7 with sodium bicarbonate. 240 fish were split into two groups for injections: cortisol/carrier and carrier only. The average fish weight was 31.01 grams (n=10); a dose of 0.01 mg cortisol/g body weight of fish should elevate plasma cortisol levels to 60 ng/ ml. Pelvic fin clips were used to distinguish between groups and were administered directly following injection.

Sampling:

Recovery was monitored before transferring to tanks at the SDL. Each group (cortisol injected and carrier only) was split evenly into four 0.91 m diameter tanks, so that each tank had 120 fish, 60 cortisol injected and 60 carrier only. Four days after the injection of cortisol, 608 Juga snails (*Oxytrema silicula*) each were added to two of the tanks. Snails had been collected from the Willamette River and tributaries (Marys' river). A subsample of snails was screened for infection with *N. salmincola*. To do this, snails were placed in individual petri dishes overnight with water and organic lettuce. Petri dishes were examined for *N. salmincola* cercaria the following morning with a dissecting microscope.

The experiment ran for a total of 51 days and fish were sampled about every two weeks to determine infection and cortisol levels. Each sampling date, twelve fish per tank were euthanized with 250 mg/L MS-222 buffered to a pH of 7 with sodium bicarbonate and samples were taken as described below. On the last sampling date ~ 40 fish per tank. Additionally, 30 of the juvenile fish were sampled at the FPGL over the course of the experiment to determine baseline cortisol levels.

Blood/tissue processing:

Blood was collected from the caudal vasculature using heparinized Natelson tubes. Blood was then transferred to a 500 ul centrifuge tube and kept on ice until centrifuged. Plasma was drawn off and stored at -80 ˚C. Radio-immuno assays were done to determine blood plasma levels of cortisol (Redding 1984). Gill, heart and kidney samples were taken from each fish to determine infection level by wet mounts (as described in chapter 1).

Results

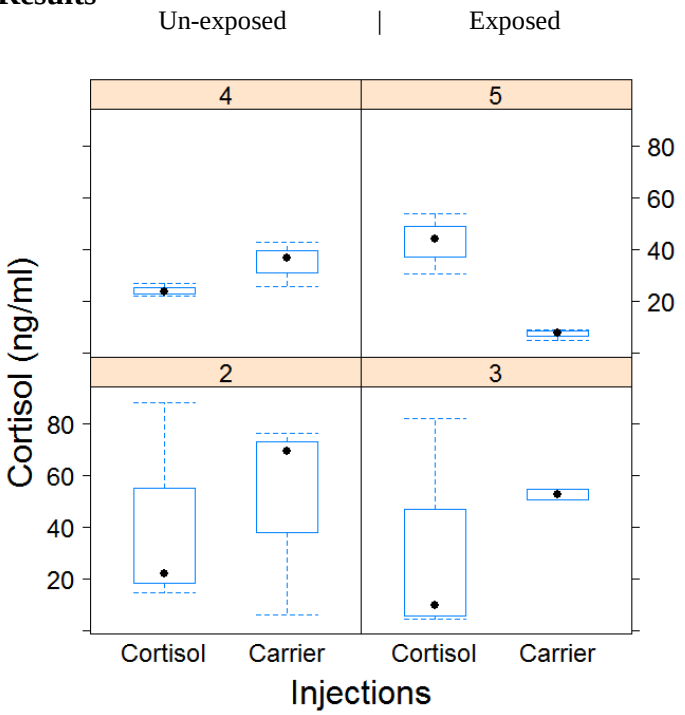


Figure 1. Cortisol (ng/ml) by injection group for each tank. On the x axis, Cortisol means fish were cortisol treated and Carrier indicates fish were injected only with cocoa butter. Data is from the first sampling date (Week 2: 8/14/12). Panels numbers correspond to tank numbers; Tank 5 and 3 were exposed to *N. salmincola*, tanks 4 and 2 were not exposed.

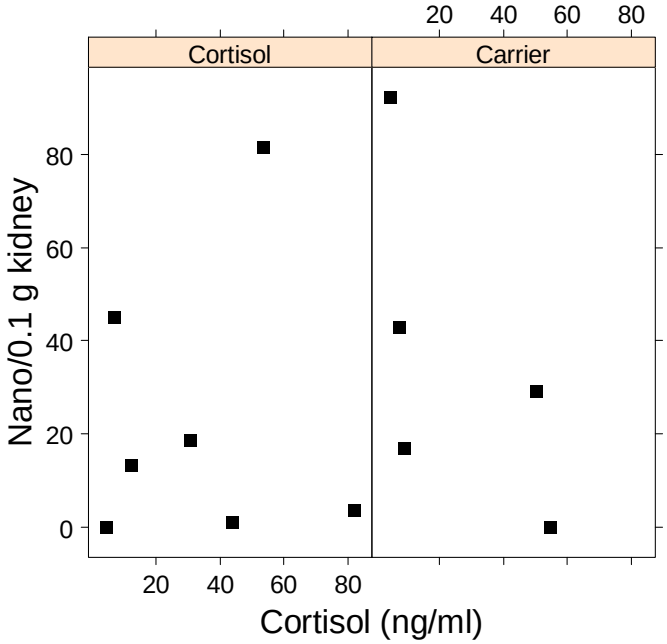


Figure 2. Number of *N. salmincola* per 0.1 g kidney by cortisol (ng/ml) from the first sampling date (Week 2: 8/14/12). The graph is paneled by the injection fish received: cortisol or carrier only(cocoa butter). Includes only fish exposed to parasites.

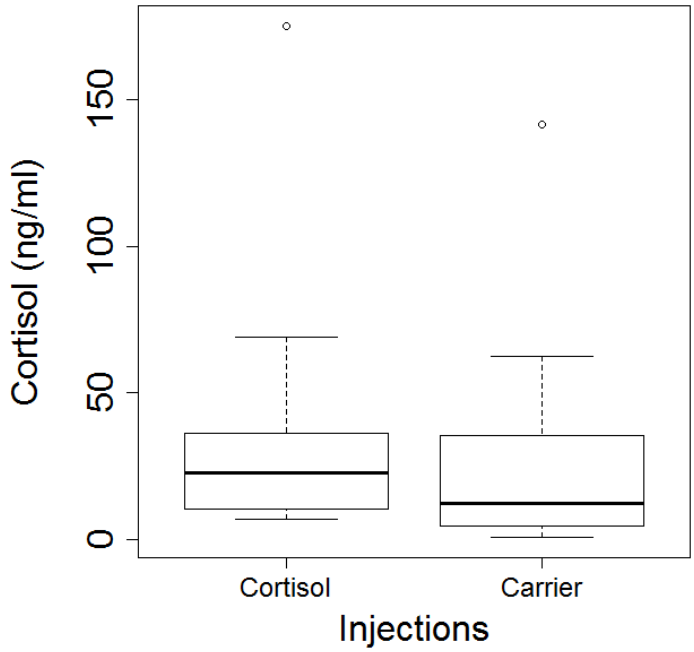


Figure 3. Mean cortisol (ng/ml) in fish injected with cortisol (cortisol) and injected with vehicle only (carrier) for tank 3 at last sampling (Week 8:9/20/12).

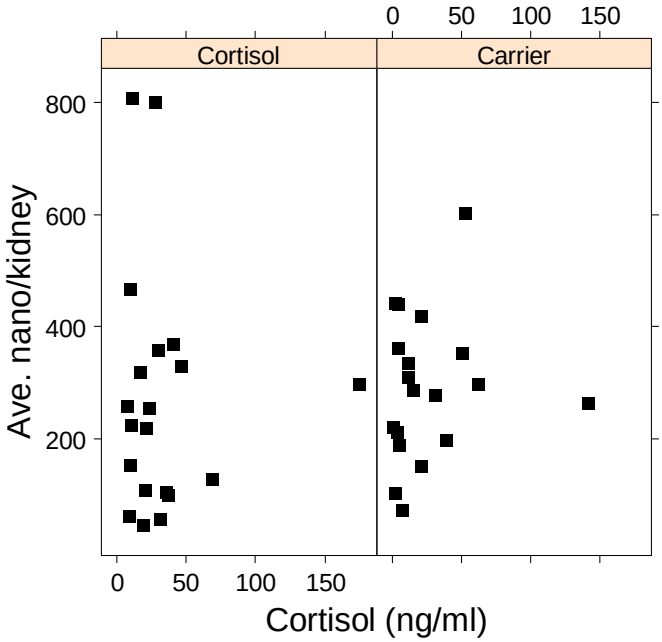


Figure 4. Average counts of *N. salmincola* per kidney by cortisol(ng/ml) for tank 3 (Week 8: 9/20/12). The graph is paneled by injections received: cortisol and carrier only.

There did not appear to be any difference in cortisol concentrations between those fish injected with the steroid and those receiving only the vehicle (Fig. 1). I expected to find elevated levels in the fish treated with the hormone. Given that there was no clear evidence that fish experienced elevated cortisol, I regressed infection severity against individual cortisol level to see if there was any correlation. For the earliest sampling date, there was no correlation between the number of parasites in kidney and cortisol levels (Fig. 2; n=12, anova, p value =0.4529).

By the last sampling date, cortisol levels were low regardless of group (Fig. 3). As seen in Fig. 4, again there was no correlation of parasites and cortisol levels (n= 39, anova, p value=0.9313). Note that for this date, *N. salmincola* counts were not standardized to 0.1 g kidney. The six I did standardize from this tank and the 15 from the 2nd tank still did not show a difference between groups (based on fin clip alone).

Discussion

While I did not see the expected results, I cannot be sure if this is due to no effect of cortisol on susceptibility, or if cortisol was not high enough to see an effect. Cortisol injected with a cocoa butter carrier should result in a slow release of cortisol---for a high level of cortisol for a period of about 5 weeks. On the first sampling date, cortisol levels were variable and lower than predicted, with only one tank showing a distinct separation between cortisol and carrier only injected fish. Fish received a 100 ul injection of cortisol, that should have risen cortisol to 60 ng/ml in a 30 gram fish (the average weight of fish in this study) based on previous work (Maule et al. 1987). There was no correlation of cortisol with weight, so it is unlikely that giving all fish 100 ul injection caused the variation in cortisol levels.

Levels of cortisol in the control group were also much more variable than predicted. Baseline cortisol levels taken from fish held at the FPGL ranged from 5 to 25 ng/ml (n=6), whereas carrier only injected fish had levels ranging from 5 to 76 ng/ml (n= 11) for week 2. It is possible that the injections themselves were mildly stressful, which has been shown by (Wang et al. 2005, Harris et al. 2000, Richman and Zaugg 1987). However, even with a slight increase for carrier injected fish, these papers all had distinct groups (i.e. cortisol injected fish were still much higher than carrier injected fish).

One source of variation likely comes from location; baseline cortisol levels were not done at location of experiment. It is possible that fish held at SDL have a higher average in baseline cortisol. If this is true, then a higher dose of cortisol would have to be injected to create two distinct groups.

In all groups, variation could be also explained by the fishes' ability to clear out cortisol---some were probably faster than others. Finally, it is possible that some fish may have been incorrectly fin-clipped, but it is highly unlikely that this could have happened to the extent that would lead to this much variation in cortisol levels.

Knowing whether cortisol increases susceptibility to infection of a non proliferative pathogen has two important impacts. First, if cortisol does have an effect on susceptibility, then I would argue that non proliferative pathogens are just as important as proliferative to an immune compromised host. If infection with non proliferative pathogen is not correlated with cortisol, then possibly *N. salmincola* could serve as a marker—a fish with this level of infection has probably been in the river for X number of days. In addition, knowing the answer to this question shapes management decisions. If cortisol is playing a role, management should focus on reducing human impact on the stress of migration. For instance, holding regimes could be adjusted to try to reduce stressors such as handling and crowding. Fish could be held in pathogen free, constant temperature environments before release, reducing exposure to high temperatures, parasites, and other stressors.

Future exploration of this topic would benefit greatly from three things. The first would be to run a pilot test of cortisol injections. Fish should be held at the location the experiment will be performed, injected with a higher dose (200 ng/ml cortisol, within the physiological realm of spring Chinook salmon), and cortisol should be tested weekly over the proposed duration of the experiment (e.g. 8 weeks). Secondly, once the cortisol dose has been determined, it would be better to sample an equal number over the course of the experiment. This way if the effect happens early on in the experiment, one will have a higher sample size to detect it. Finally,

another option would be to remove fish from snails over the course of the experiment and allow additional time for the parasite to encyst in the kidney.

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Appendix C: Testosterone levels in female spring Chinook salmon

Introduction

Testosterone is correlated with reproductive maturation in female coho salmon and spring Chinook salmon, with fish showing an elevation in testosterone levels as they get closer to ovulation (Fitzpatrick et al. 1987; Slater 1991). We hypothesized that if female spring Chinook salmon had higher levels of testosterone earlier, this could be indicative of premature senescence.

Methods

Blood collection and processing:

Spring Chinook salmon were sampled at Willamette Falls as well as Foster, Dexter, Fall Creek, and Minto traps. In addition, blood was collected from spawned spring Chinook salmon at the Fish Performance and Genetics Laboratory in Corvallis, OR. Blood was taken from caudal vasculature using heparinized vacutainers within 3 minutes of death (or capture for radio tagged fish) (Maule et al. 1996). Samples were held on ice until they could be centrifuged for eight minutes. Plasma was drawn off and stored at -80 °C.

Testosterone concentrations were determined by radioimmunoassay as in Fitzpatrick et al. (1987). The steroid was extracted using diethyl ether, reconstituted in phosphate buffered saline-gelatin solution, and the assay was run following protocol. Each sample was spiked with tritiated testosterone so that extraction efficiency could be determined. Based on work by Slater (1991), testosterone was assessed only for females only, as males do not show an increase of testosterone with time.

Results and Discussion

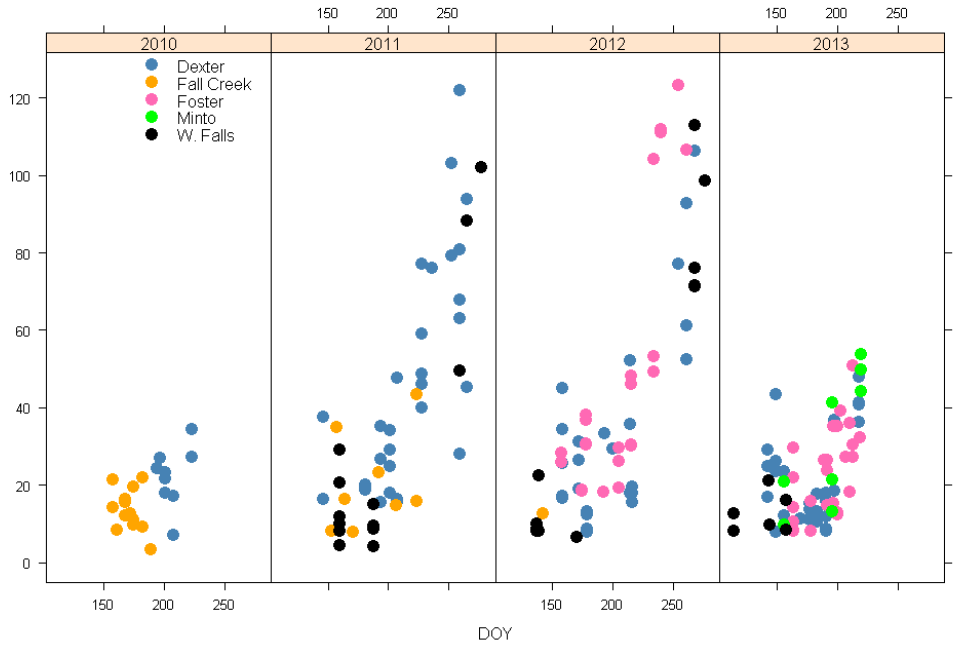

Figure 1. Testosterone (ng/ml) for each fish by day of year (DOY), paneled by year. Each color represents a different location; W. Falls is Willamette Falls. Fish spawned at the Fish Performance and Genetics Laboratory are included in this graph.

Figure 1 depicts the testosterone levels of each fish by day of year, paneled by year. In each year(2010-2013), there is an increase in testosterone levels with an increase in time (DOY). While there is variation between fish for a given day of year, these results are in agreement with Slater (1991). However, higher testosterone levels were not seen earlier in the year, indicating that these female Chinook salmon are not reproductively mature earlier than normal.

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Appendix D: Interrenal cell nuclei as an indicator of cortisol production.

Introduction

As salmon start the last stage of their life, they lose the ability to regulate cortisol (Robertson et al. 1963), a hormone that plays a role in immunity, stress, growth and reproduction. Cushings' syndrome is the condition when vertebrates are unable to down-regulate cortisol, attributable to both increased secretion and decreased clearance (Dorland's Illustrated Medical Dictionary 1974). If salmon are experiencing elevated levels of cortisol earlier than they should, (thus speeding up the senescent pathway), this may be an underlying cause of prespawn mortality.

Cortisol elevates rapidly following a stressor, which is why samples are taken within 3 minutes of capture to reduce the influence of capture stress (Schreck & Moyle 1990). It is likely that the trapping facilities are stressful and we do not get a baseline level of cortisol from fish sampled at these locations. In an attempt to get a more stable view of cortisol production, it was decided to look at interrenal cells. Interrenal cell nuclei increase in size with an increase in cortisol production (Fagerlund et al. 1968). Another benefit of looking at interrenal nuclear diameter is that the fish does not have to be alive. Samples can be collected from freshly dead fish, which is helpful when dealing with prespawn mortalities.

If prespawn mortality is a result of premature senescence, I would expect to see larger diameters of interrenal cell nuclei in prespawn mortalities than in cohorts.

Methods

Anterior kidney samples were fixed in 10% neutral buffered formalin, then sectioned and stained as in chapter one and Sloman et al. (2000). Slides were scanned at 10X magnification for interrenal cells. 40X magnification was used to confirm cells. Briefly, the anterior kidney contains three types of cells: interrenal, chromaffin and hematopoetic cells (Nandi 1962). Interrenal cells are generally located around the post cardinal vein.

Image analysis software (Image Pro Plus) was used to measure the diameter of interrenal cell nuclei at 63X magnification. 10 cells were selected per field, and 3 areas of interrenal cells per fish were examined if possible. Average nuclear diameter(μm) was calculated per fish. Nuclear

size was then compared between fish sampled at trapping facilities, prespawn mortalities and spawned fish (at holding facility and outplanted).

Results

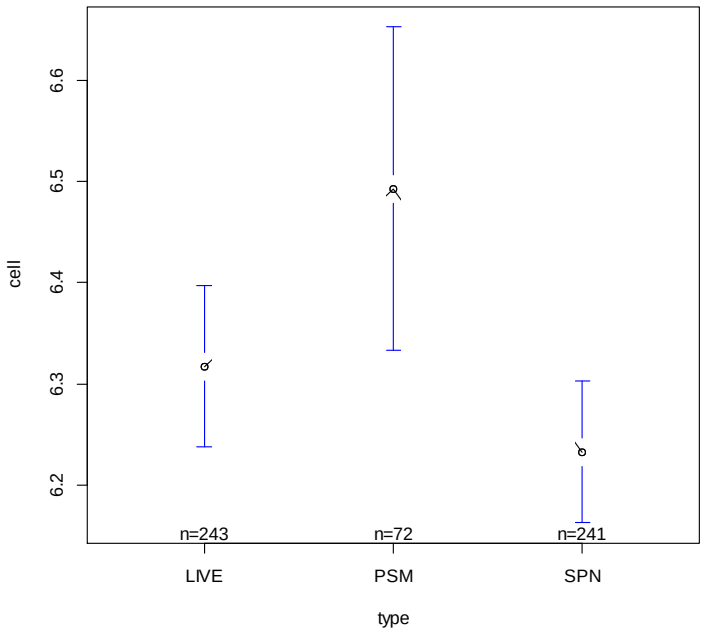

Figure 1. Mean (+/- 95% CI) nuclear diameter(μm) of interrenal cells for fish sampled at trapping facility (LIVE), PSM, and spawned fish (SPN). n indicates the number of cells measured for each group (not the number of fish).

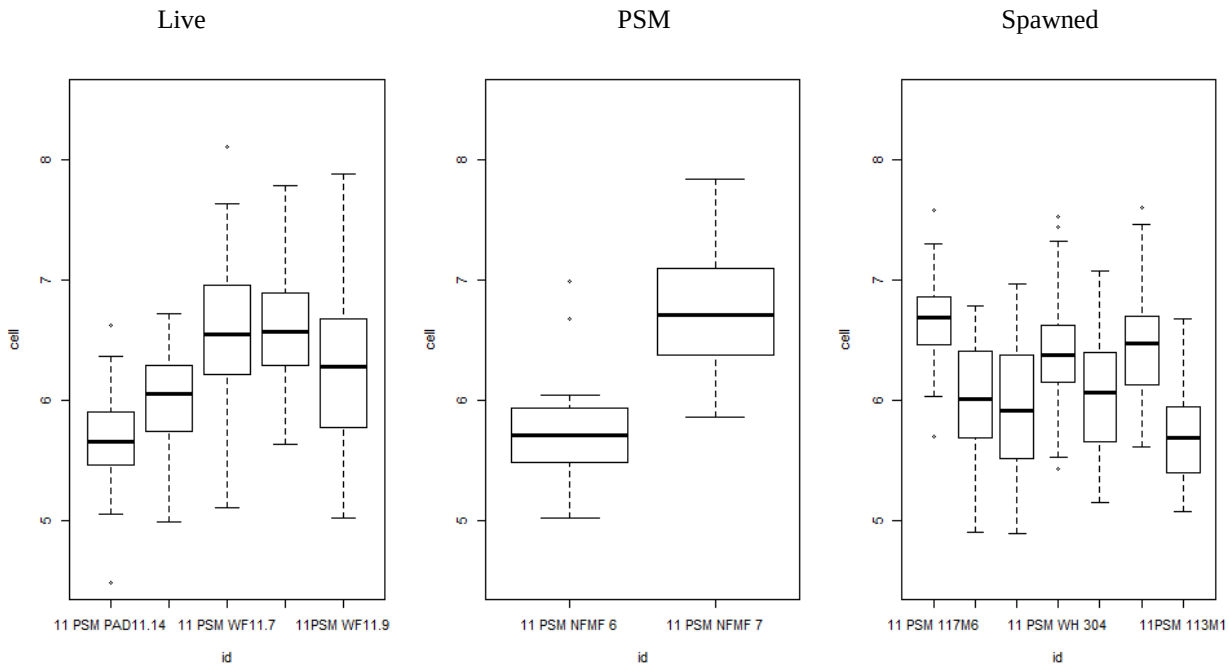

Figure 2. Mean nuclear diameter (μm) of interrenal cells for each fish sampled at trapping facility (Live), PSM, and spawned fish.

Table 1. Mean diameter (μm) and SD of interrenal nuclei for each fish. ID, location, fish type (PSM, spawned, live), collection date, number of cells measured and sex of each fish are also provided. Live indicates fish sampled at the trapping facility.

					Diameter (μm)	
Type	id	date	sex	# cells measured	mean	SD
PSM	11 PSM NFMF 6	7/21/2011	M	20	5.77	0.52
	11 PSM NFMF 7	8/24/2011	F	52	6.77	0.51
SPN	11 PSM WH 11.21	9/13/2011	M	31	5.92	0.55
	11 PSM WH 304	9/13/2011	F	60	6.39	0.47
	11 PSM WH11.298	9/13/2011	F	30	6.02	0.51
	11 PSM WH11.513	9/20/2011	F	38	6.47	0.45
	11PSM 113M1	9/22/2011	M	24	5.72	0.42
	11 PSM 117M6	10/3/2011	M	34	6.67	0.40
	11 PSM FC 28	9/22/2011	M	24	6.01	0.46
Live	11 PSM WF11.11	5/28/2011	F	32	6.00	0.40
	11 PSM WF11.7	5/28/2011	F	69	6.62	0.55
	11PSM WF 11.12	5/28/2011	F	50	6.63	0.48
	11PSM WF11.9	5/28/2011	M	61	6.23	0.65
	11 PSM PAD11.14	9/27/2011	M	31	5.66	0.41

Over 200 samples were collected, but only 25 samples had interrenal cells. From the 25, only 2 were prespawn mortalities, one of which died shortly after transport (Table 1, NFMF 6). I then chose 7 spawned fish and 5 fish sampled at trapping facilities(live). Table 1 provides basic information on each fish examined. Figure 1 shows the mean interrenal nuclear diameter (μm) for PSM, spawned and live groups. Figure 2 shows mean interrenal nuclear diameter (μm) for each fish.

Discussion

That I was unable to locate interrenal tissue despite being the correct region of kidney is interesting. Robertson and Wexler (1960) reported the adrenals in adult salmon are hypertrophic, indicating that interrenal cells should still be in existence. In Pacific salmon, the anterior kidney branches into two lobes (i.e. the kidney looks like a Y), and both sides contain interrenal cells (Nandi 1962). In other words, sampling only one lobe should be effective. Hematopoietic tissue was visible in all samples, indicating I was in the right area of the kidney. I had serial sections done for some samples that had hematopoietic tissue; interrenal cells were still not sighted in these sections.

Future work might consider in vitro testing of interrenal cells' ability to function. Or if this were to be re-done, consider taking both sides of anterior kidney, and cut section so that blood vessel (which interrenal cells should be located around) is visible on slide.

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Appendix E: Transmission of *Parvicapsula minibicornis* by intraperitoneal injection of infected kidney tissue.

Introduction

P. minibicornis is a proliferative pathogen that is associated with prespawn mortality in sockeye salmon of the Fraser river (St-Hilaire et al. 2002; Wagner et al. 2005; Bradford et al. 2010). It is also seen in Willamette River spring Chinook salmon, and has been indicated as the cause of mortality in some cases.

The current methods of studying this parasite is to collect fish known to be infected or expose fish to river water that is known to have parasite. The main drawback to these methods is the inability to prevent co-infections with other pathogens. It would be helpful for laboratory experiments to have an alternative method of infection. Previous work has shown that *P. minibicornis* infects juvenile salmonids (St-Hilaire 2002). Injection of fish with pathogens has been successfully done with *Aeromonas salmonicida*, *Yersinia ruckeri*, and Proliferative Kidney Disease (Bullock et al. 1976; Feist and Bucke). The latter is a myxozoan, similar to *P. minibicornis*. We injected infected salmon kidney tissue into juvenile spring Chinook salmon to determine if this technique will successfully transmit *P. minibicornis*.

Methods

About 3 inches of posterior kidney was collected from each of 7 adult spring Chinook salmon at Dexter trapping facility on Willamette river, OR. Kidneys were individually stored in whirl packs with tissue culture media (with 2X antibiotics) and held on ice.

Tissue smears were prepared to check for the presence of P. minibicornis and the absence of bacteria. Samples with the desired pathogen were combined and suspended at a ratio of 1:4 tissue to sterile Phosphate Buffered Saline (PBS). The sample was then macerated and passed through a 100 um cell strainer to prevent clogging the syringe needle. Solution was held in ice until injection.

For intra-peritoneal injection with infected kidney tissue, fish were anaesthetized with 50 mg/L buffered (pH of 7) MS-222. 60 spring Chinook salmon (average weight ca 30 g), were injected

with 500 ul of the solution using a hypodermic syringe (20 g needle). After injection, fish were evenly divided into three 100 L tanks and watched for recovery. No fish died from injections.

For the length of the experiment, fish were held in 13 °C water at the SDL and fed commercial salmon feed daily following standard operating procedures. Fish were examined at least twice a day for any signs of morbidity. Fish showing signs of lethargy, ataxia, dermal lesions, hemmorrhaging, or rapid breathing were removed and euthanized immediately with an overdose of buffered MS-222.

15 fish from each treatment were sampled four times (every 22 days) over a 3 month period. Fish were euthanized with an overdose of buffered MS-222. The entire kidney was fixed in 10% neutral buffered formalin. After 7 days of fixation, kidney samples were embedded, sectioned and stained with H& E by Vet diagnostic lab. Mike Kent examined the slides for *P. minibicornis*.

Results and Discussion

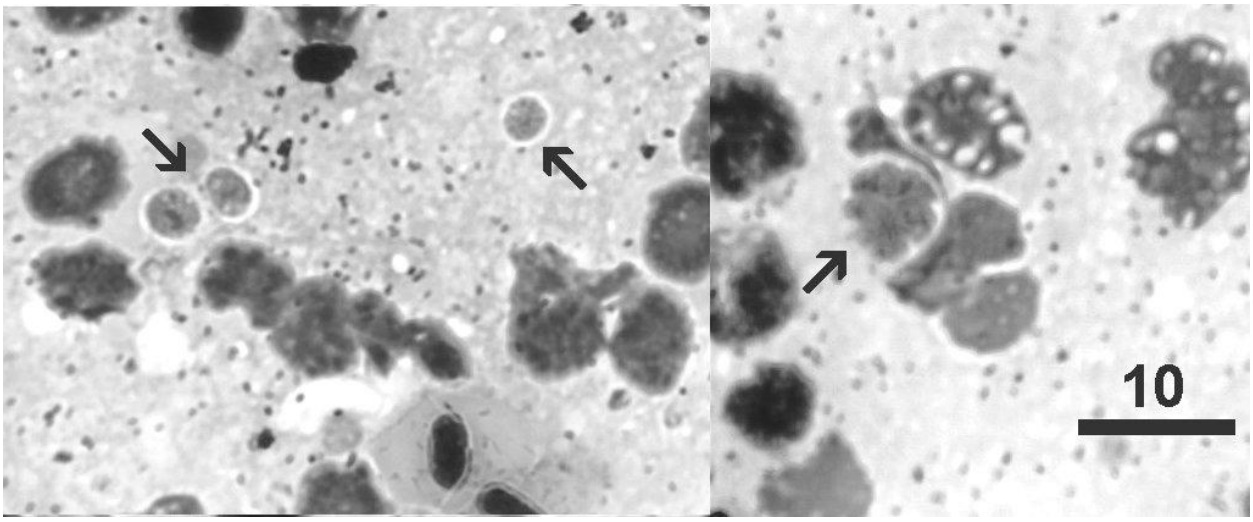

Figure 1. *Parvicapsula minibicornis* in adult spring Chinook salmon kidney. The arrows indicate unicellular (left picture) and multicellular(right picture) myxozoan extrasporogonic forms.

Parvicapsula minibicornis was present in the adult kidney tissue used for inoculum, as determined by both kidney smears(Figure 1) and histology. However, *P. minibicornis* was not detected in any of the injected fish.

There are several explanations for why *P. minibicornis* was not transmitted. Perhaps the length of time between collection of tissue and injection was too long, or maybe the temperature was not favorable for *P. minibicornis*. Based on histology, *P. minibicornis* in the adult fish was only present in the glomerulus; perhaps this is not an infectious stage. This parasite is known to infect juveniles, therefore differences in life stage is not likely explanation. Finally, it is possible that injection of infected kidney tissue is not a viable method of transmission.

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