Puntledge River Summer Chinook Parentage-based Tagging Study Year 3

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Prepared for:

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EXECUTIVE SUMMARY

Genetic analysis methods will be used in this multi-year study to identify individual Puntledge River summer-run Chinook salmon back to parental crosses (both those that were performed in the hatchery and those that occurred in the wild) to study the effects of parental Chinook return migration time and BKD status on their progeny. The genetic analysis is known as 'parentage-based tagging' and it allows identification of an individual offspring (at any age, including adults) to its parental pair, as long as both parents have been sampled and genotyped. The genotyping of parents and offspring will be conducted with a set of fifteen microsatellite loci (genetic markers) that are analyzed in the Molecular Genetics lab (MGL) at the Pacific Biological Station. Fisheries and Oceans Canada (DFO) considers the Puntledge River summer-run Chinook salmon a population of high conservation concern. This research will provide information on the most effective strategies to implement in re-establishing successful reproduction both in the hatchery and in the wild. This project is identified under the Species Based Actions in Table 2 of the Salmonid Action Plan – Support hatchery activities focused on enhancing the earliest returning adults of the summer Chinook run - which is ranked as a Level 1 priority.

To date, high quality DNA extracted from adult summer Chinook salmon sampled from the Puntledge River in 2013 - 2015 has been analyzed. Tissue samples from 211 brood year 2015 summer Chinook adults sampled from the natural environment above the Puntledge diversion dam, the principle summer Chinook spawning grounds, and 702 naturally-spawned juveniles collected between February and July 2016 during outmigration were successfully analyzed. The 211 natural spawners transported to Comox Lake were estimated to be approximately 59% of the Summer Chinook natural spawners that emigrated past the hatchery brood collection point in the lower river during 2015.

DNA extraction and analysis of brood year 2016 hatchery and natural spawners is underway in the Molecular Genetics Lab at the Pacific Biological Station. Also being analyzed are 147 and 700 jack samples collected in the adult returns of 2015 and 2016. A subset of these returns are believed to be progeny from the 2013 and 2014 hatchery brood fish and natural spawners. The genotypes from the jack samples will be used to test our ability to assign returning progeny back to a unique pair of parents in our spawner DNA database.

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1 INTRODUCTION

This is year three of a multi-year project that will utilize genetic analysis methods to identify the Puntledge River summer-run Chinook salmon parents of first generation adult returns (the parents that were originally spawned in the hatchery and those that spawned in the wild) to investigate the heritability of migration timing and bacterial kidney disease (BKD). The genetic analysis used in this study is known as 'parentage-based tagging' (PBT) and it allows identification of an individual offspring (at any age, including adults) to its parental pair, provided that both parents were originally sampled and genotyped. The offspring will be sampled as downstream migrating juveniles or as returning adults over the next one to four years. The BKD status (negative or low-positive) of female hatchery parents and the migration timing of both male and female parents will be compared with the survival and migration timing in their offspring to determine the influence of female low level BKD infection on offspring survival and the heritability of migration time in hatchery- and naturally-spawned Puntledge summer Chinook salmon.

While the enhancement of the summer Chinook population at the Fisheries and Oceans Canada's Puntledge River Hatchery has stabilized, levels remain below DFO's target escapement. The current research will provide information on the most effective strategies to implement in re-establishing successful reproduction both in the hatchery and in the wild. The project has strong support and cooperation between DFO, the K'ómoks First Nation and BC Hydro and will provide information to guide the efforts of stakeholders in restoring the salmon populations in the Puntledge River that have been impacted by hydro development. This project is identified under the Species Based Actions in Table 2 of the Salmonid Action Plan – Support hatchery activities focused on enhancing the earliest returning adults of the summer Chinook run - which is ranked as a Level 1 priority (FWCP 2011).

1.1 Background

Access and utilization of habitat above BC Hydro's diversion dam is critical to the sustainability of summer Chinook and coho salmon production in the Puntledge watershed. Past studies on summer Chinook migration in the Puntledge River have indicated that summer Chinook adults that arrive in the lower Puntledge River prior to July have a greater success migrating to the upper river (at or above the diversion dam) compared to those that arrive later in the summer (95% versus 50% success rate). The

success of early arriving fish is attributed to cooler migration temperatures in the river, low recreational use, and the higher availability of spring freshet spills that aid upstream Chinook migration into Comox Lake. In contrast, later arriving Chinook must contend Kwith warmer river temperatures, lower flows, and a high level of disturbance from swimmers, particularly at Stotan and Nib falls, two areas that present some of the greatest challenges for migration. Furthermore, studies have also shown that Chinook that are able to hold in the cooler depths of Comox Lake throughout the summer have a spawning success rate of 95% compared to ≤ 50% for fish that hold below the diversion dam (Guimond and Taylor 2010).

This clearly demonstrates that the most productive strategy for summer Chinook adults is to migrate into Comox Lake early (i.e. before July), hold in the lake during the summer and then spawn above the diversion dam at the lake outlet (headpond) or in the two main Comox Lake tributaries (Upper Puntledge and Cruickshank rivers).

The Puntledge Hatchery Salmonid Enhancement Program (SEP) has incorporated these watershed species requirements into their Production Strategy. A higher proportion of the earlier returning summer Chinook are utilized for hatchery broodstock which is expected to re-build the earlier component of the summer Chinook returns thus improving migration success to the upper watershed. If the early returning behaviour is genetically controlled, selecting earliest returning adults for brood and mating them with each other should result in an earlier returning summer Chinook in the following generation. It is anticipated that, over time, this strategy will have the following benefits:

- increase the separation in migration timing between summer and fall Chinook,
- increase the success of summer Chinook salmon returning and migrating to the upper watershed and Comox Lake,
- increase the number of successful spawners above the diversion dam while reducing the number that remain in the lower river, and
- reduce the risk of hybridization between summer and fall Chinook.

All hatchery activities that enhance the earliest returning adults of the summer Chinook run are identified as a high priority “Species Based Actions” in the FWCP-Coastal Salmonid Action Plan for the Puntledge watershed (FWCP 2011).

1.2 Goals and Objectives

The overall goal of the study is to provide guidance for the development of appropriate hatchery protocols that will maintain the genetic distinction of the summer and fall Chinook populations, properly manage BKD in the summer Chinook population

and optimize the survival. A thorough understanding of these two factors, as described below, will be critical to the rebuilding efforts of the Puntledge River summer Chinook population.

i. **Assessment of run timing inheritance -** Migration time has been shown to be genetically controlled (and therefore heritable) in Chinook salmon (Healey 1991). Moreover, the returning progeny from early- or late-migrating parents tend to return at similar times. Therefore, we expect the early (May-June) and late (July -August) migrating adults spawned in the hatchery, and those that spawn in the wild, to produce offspring with similar adult migration timing. However, environmental factors (e.g. marine conditions, freshwater temperature and flow levels) also affect migration and introduce annual variation in migration timing (Anderson and Beer 2009). This study will enable us to calculate the degree of genetic and environmental influences on migration time in Puntledge summer Chinook salmon and the degree to which selection for early migration times may be effective in improving their survival and abundance. Selection for early migration time in the summer Chinook has the added benefit of facilitating genetic separation between the summer and fall Chinook salmon populations within the Puntledge drainage; maintaining this genetic distinction is necessary for adaptation and long-term conservation of the summer run.

ii. **Assessment of BKD resistance** - *Renibacterium salmoninarum*, the causative agent of BKD, is an endemic pathogen in the Pacific Northwest. BKD is a slowly progressing, lifelong infection of salmonids. The bacterium may be horizontally transmitted between fish and vertically transmitted to the next generation. Fish infected with *R. salmoninarum* will not normally exhibit clinical signs until the fish are a year old. As such, BKD is a serious disease in salmon culture. From a husbandry perspective, good hatchery practice is to eliminate or minimize presence of the pathogen in the hatchery (and subsequently the natural) environment by culling progeny from BKD-positive female parents. However, there may be a genetic disadvantage to this practice if, in fact, the positive females that are being selected against carry genes that enable tolerance of the pathogen and the ability to survive and reproduce, even in the presence of bacterial infection.

The fate of hatchery individuals that are disease-free but carry the *R. salmoninarum* bacterium due to vertical transmission, and their impact, or lack of, on their naturally-spawned counterparts following release has not been closely studied. Good husbandry and a precautionary approach to wild interactions have been the driving factors to date in developing appropriate protocols for responding to infection in the hatchery environment. However, it is important to determine if exclusion of all progeny from *R.*

salmoninarum positive females is hindering inherent mechanisms of BKD resistance in some populations, as well as possibly contributing to an unnecessary loss of genetic diversity in populations of conservation concern. Conversely, investigating the putative role of immunotolerance and heightened BKD susceptibility following vertical transmission of the pathogen will inform future DFO SEP BKD management strategies. The ability to follow the survival and reproductive success of offspring from individual BKD positive and negative females in the Puntledge summer Chinook population will assist both in its management and in the refinement of general husbandry protocols for BKD affected hatchery populations.

A secondary objective of the study is to examine the genetic diversity in the natural spawning population and determine if the genetic diversity present in the adult population is being effectively transmitted to the juvenile stage of the next generation. This will provide insight into the future success of rebuilding a sustainable Puntledge River summer Chinook population.

2 STUDY AREA

The Puntledge River Watershed encompasses a 600 km^2 area west of the city of Courtenay (Figure 1). The lower Puntledge River flows from Comox Lake in a north-easterly direction for 14 km where it joins with the Tsolum River. From this point downstream the river is called the Courtenay River, and flows for another 2.9 km into the Strait of Georgia. The lower river below Comox Lake is divided into 3 major reaches. Reach B, the headpond reach, is located between the Comox impoundment dam at the outlet of Comox Lake, and the Puntledge diversion dam approximately 3.7 km downstream. Reach C, the diversion reach, extends downstream of the diversion dam for 6.3 km to the BC Hydro Puntledge Generating Station or “Powerhouse”. Reach D encompasses the remaining 4 km of the Puntledge River from the Powerhouse to the Tsolum River confluence. Puntledge River Hatchery is located 400 m downstream of the Powerhouse. A barrier fence across the river directs migrating fish into a fishway where they may proceed further into concrete raceways in the facility, or continue their migration upstream in the river depending on the hatchery’s broodstock collection requirements.

The Puntledge River system is one of a few rivers on the east coast of Vancouver Island that supports both a summer and fall-run of Chinook salmon. The two runs have discrete migration timings and spawning distributions in the river. Summer-run Chinook enter the river from May to August while fall-run Chinook enter from September to

October. However both stocks spawn at the same time, from early October to early November.

Puntledge summer Chinook are genetically distinct from the fall Chinook stock. It is surmised that the summer-run evolved from early migrants of an ancestral fall-run stock that were able to ascend two large waterfalls in the lower river (Stotan and Nib falls) during the natural spring freshet period between April and June/July, and hold in Comox Lake prior to spawning. The two partial obstructions were once critical in maintaining the spatial segregation and genetic integrity of the two stocks. Today, both summer- and fall-run Chinook may access spawning habitat above these waterfalls, while only summer Chinook are permitted access to their historic spawning grounds upstream of the Puntledge diversion dam.

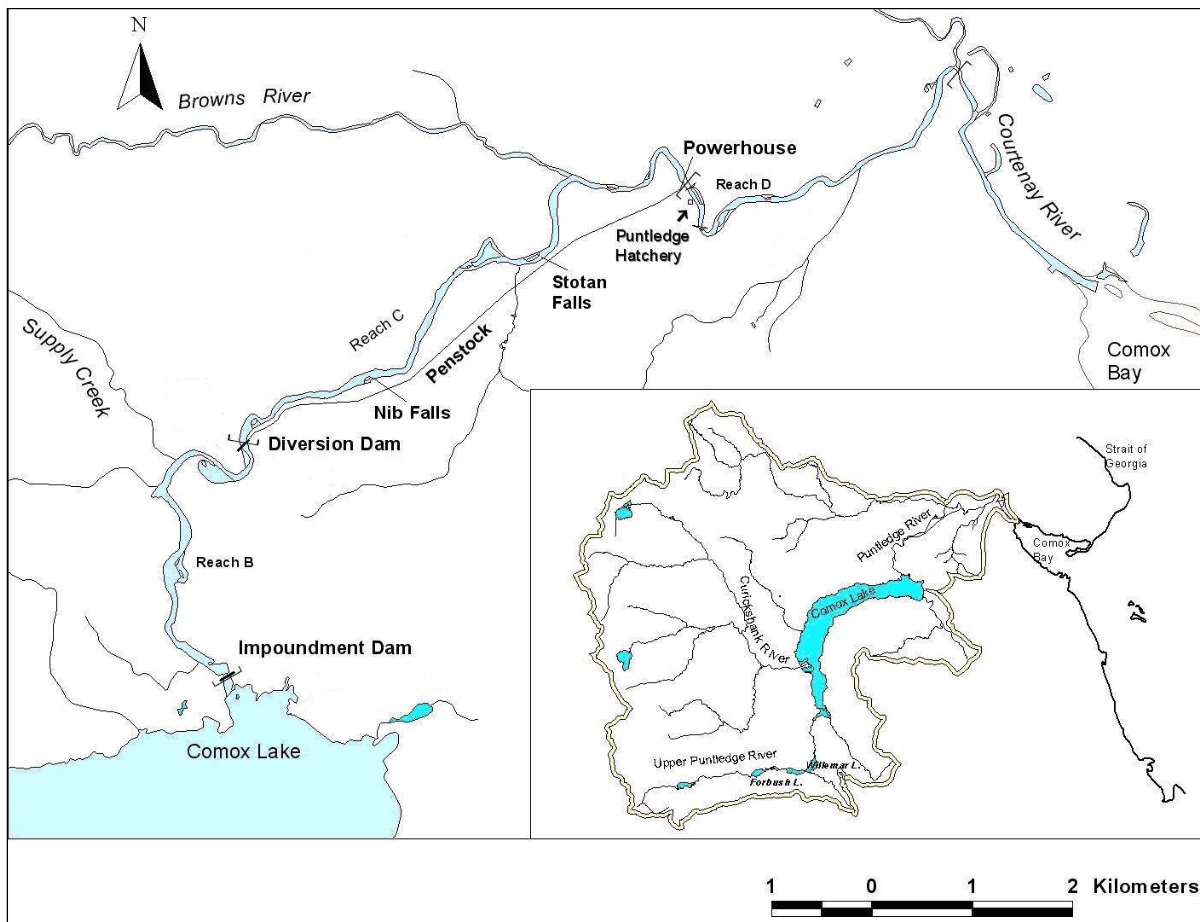

Figure 1. Location map of the Puntledge River watershed and lower river features.

3 METHODS

3.1 DNA tissue sampling of summer Chinook salmon hatchery broodstock and wild summer Chinook salmon spawners at Puntledge River Hatchery

The overall goal is to obtain high quality DNA tissue samples and accurate biological data from all broodstock spawned at the hatchery, and 50-70% of the adults that spawn in the wild.

Summer Chinook adults migrating into the Puntledge River were diverted at the barrier fence into raceways at the lower Puntledge River Hatchery beginning in mid-May 2016. Every few days the adults were crowded for sorting and transport. Adults retained for broodstock were loaded onto a transport tank containing chilled water (typically 4-6 ^{o}C cooler than ambient) and transported to Rosewall Creek Hatchery. The remainder were transported and released directly into Comox Lake where they could hold over the summer and spawn naturally in the fall. Prior to transport, a tissue sample was collected from the caudal fin using a standard paper hole punch, and affixed to a Whatman tissue sample sheet for DNA analysis. Care was taken to avoid contamination during sampling by rinsing sampling tools in water and wiping with a paper towel in between each tissue sample.

Summer Chinook broodstock transported to Rosewall hatchery were held in separate rearing tubs over the summer, based on their arrival time at Puntledge Hatchery. Those arriving prior to July 1st were treated as "early" timing fish and those arriving between 18 July and 2 Aug as "late" timing fish. In addition, a mid-timing group of adults from 2-13 July were also held separately. The broodstock in each group were spawned within their own timing group over 4 egg collection periods or Lots between 5 and 26 October 2016. During each egg-take, males and females from each spawning pair were DNA sampled, measured for length (postorbital-hypural) and scale sampled. In addition, a kidney tissue sample (for BKD analysis) was collected from each adult spawned and placed in separate Whirl-pak® bags with corresponding ID #s. BKD sampling followed specific procedures outlined in the Puntledge River Hatchery Fish Health Management Plan.

Each tissue sample collected was labeled with a unique ID #, and used to track the samples that were DNA analyzed with other corresponding biological data including sex, date of river entry (or arrival at Puntledge Hatchery), date sampled/spawned, length, tag information, markings, and BKD screening results (hatchery broodstock only). The associated data was reviewed at the lab to ensure accurate information was recorded for every fish sampled. Any discrepancies were resolved by hatchery staff before samples

were analyzed. DNA samples from broodstock whose eggs were culled were not genotyped.

3.2 Juvenile summer Chinook sample collection

The evaluation facility at the Puntledge diversion dam was used to collect a portion of downstream migrating summer Chinook juveniles from natural spawning above the dam. DNA analyses of juvenile Chinook tissue samples will allow us to assess the spawning success and mate choice of hatchery fish that returned to spawn in the wild, and will provide insight as to whether infected adults returning from hatchery spawning were able to contribute to the next generation produced in the natural environment. It also provides insight into the preservation and transmission of genetic diversity from the adult population to the next generation.

Sub-samples of juveniles collected between 25 March and 29 July, 2016 were added to earlier samples of emergent Chinook fry collected from 1 February to 1 March, 2016 for brood year (BY) 2015 and sent to the MGL for DNA analysis (Table 1). To date, 49 samples of BY2016 progeny have been collected at the evaluation facility.

Chinook fry samples <60 mm fork length (FL) were euthanized in an anaesthetic overdose of TMS™ [3-aminobenzoic acidethyl ester methanesulfonate] buffered with $NaHCO_3$ prior to preserving whole in 250 ml vials with 95% ethanol (RISC 1997). For juveniles >60 mm FL, a non-lethal tissue sample (caudal fin clip) was removed, placed in vials as above, and the fish were released back to the river. Beginning in 2017, non-lethal tissue sampling was also conducted on the smaller emergent fry. Samples were collected weekly (or less) in bulk such that each vial contained no more than 25% in volume of fry.

Table 1. Brood year (BY) 2015 and BY 2016 juvenile summer Chinook DNA sampling summary.

Brood Year	Group	Number Migrants Captured	Number DNA samples
2015	Emergent SCK fry (1 Feb – 1 Mar 2016) previously reported	2080	233
2015	SCK juveniles captured at the Eicher evaluation facility (25 Mar – 29 July 2016)	3575	469
	Total	**5655**	**702**
2016	Emergent SCK fry captured at the Eicher evaluation facility to date (Jan-Mar 2017)	162	49

3.3 BKD screening

The BKD specific pathogen control plan for DFO fish culture facilities has been devised to prevent clinical BKD epizootics during hatchery rearing and to reduce the risk of disease amplification through hatchery practices. It is comparable to the control strategies employed by public enhancement facilities throughout the Pacific Northwest. The plan recommends that all known BKD 'hot' Chinook and Coho stocks be annually screened and participate in egg culling and progeny segregation based on female parental Enzyme Linked Immunosorbant Assay (ELISA) optical density (O.D.) readings of *R. salmoninarum* antigen levels. Other stocks are subjected to periodic prevalence assessment of 60 fish, to confirm BKD risk status. The Puntledge summer Chinook stock was recently identified as a high risk BKD stock during routine screening of 2009 and 2011 broodstock. As a result of the revised stock BKD risk designation, the production strategy was altered to improve biosecurity and to participate in an annual BKD broodstock screening, egg segregation and culling program. Specific biosecurity measures employed include pre-spawning antibiotic administration to females prior to egg collection, iodophor egg disinfection during water hardening, incubation in individual heath trays until broodstock ELISA results are available and culling based on levels of soluble *R salmoninarum*-antigen detected using ELISA. BKD screening ratings and recommended actions include:

- **Negative** - fertilized eggs/progeny from females that have a lower optical density (OD) value than those of the kidneys of the negative control fish. These may be used for yearling programs.
- **Low Level of Detection** - OD values <0.1 but greater than the mean negative control. LLD eggs present a low enough risk of BKD to be treated as negative.
- **Low Positive** - OD value ≥ 0.1 but < 0.25. Progeny from these eggs should be released early, as unfed fry.
- **Moderately Positive** - OD value ≥ 0.25 but < 0.6. Progeny should be outplanted as eyed eggs if rearing habitat is available downstream from the water intake of the facility, or destroyed if appropriate habitat is unavailable
- **High Positive** - OD ≥ 0.6, should be destroyed.

As indicated above, progeny from females with ELISA OD values > 0.1 are normally released as unfed fry to minimize the risk of horizontal transmission of *R. salmoninarum* and to prevent BKD epizootics at SEP facilities. However, in situations where escapement numbers are low or the prevalence of BKD in the escapement is high,

the prescribed culling recommendations may compromise production targets. Deviation from these recommended actions may be permitted if conservation concerns outweigh the ecological risks of propagating progeny from *R. salmoninarum*-positive fish, provided there is mutual agreement of the hatchery management, enhancement operations support staff and the DFO veterinarian. In 2012, the OD value threshold for yearling production for Puntledge summer Chinook was raised to 0.14 and progeny were reared without evidence of clinical BKD.

In 2015 and 2016, Puntledge Hatchery staff recorded all broodstock crosses, female *R. salmoninarum* antigen levels and parental DNA sample numbers. For Puntledge Hatchery, a secondary threshold of 0.14 OD was used to separate the Low Positive group into a Lower-Low Positive and a Higher-Low Positive group. Progeny from all BKD Negative and Low Level Detection females will be raised to 5 - 6 gram 0+ smolts; progeny from Lower-Low females will be released in the Puntledge River unfed, or as 0+ smolts, while progeny from Higher-Low Positive females will be reared at minimal densities to reduce stress, and also released early (unfed) when in-river conditions (food supply, flows) allow, and if population pre‐release screening indicates the *R. salmoninarum* infection level is suitably low. Standard biosecurity measures will be employed at all times at the facility. Eggs from Moderate Positives were outplanted in Jack Creek as eyed-eggs, while eggs from High Positive females were culled.

3.4 Microsatellite analysis

DNA for the 2015 and 2016 adult and juvenile samples were extracted from the tissue samples using the Qiagen 96-well Dneasy® procedure or chelex based method. Extracted DNA was used in DNA amplification of 15 microsatellite loci as follows: Ots100, Ots101, Ots104, Ots107 (Nelson and Beacham 1999); Ssa197 (O'Reilly et al. 1996); Ogo2, Ogo4 (Olsen et al. 1998); Oke4 (Buchholz et al. 2001); Omy325 (O'Connell et al. 1997); Oki100 (Beacham et al. 2008); Ots201b, Ots211, Ots213 (Grieg 2003); and Ots2, Ots9 (Banks et al. 1999). In general, PCR DNA amplifications were conducted using DNA Engine Cycler Tetrad2 (BioRad, Hercules, CA) in 6µl volumes consisting of 0.15 units of Taq polymerase, 1µl of extracted DNA, 1x PCR buffer (Qiagen, Mississauga, Ontario), 60µM each nucleotide, 0.40µM of each primer, and deionized H2O. The thermal cycling profile involved one cycle of 15 minutes at 95°C, followed by 30 – 40 cycles of 20 seconds at 94°C, 30-60 seconds at 47 - 65°C and 30-60 seconds at 68 - 72°C (depending on the locus). Specific PCR conditions for a particular locus could vary from this general outline. PCR fragments (microsatellite alleles) were size fractionated in an ABI 3730 capillary DNA sequencer, and genotypes were scored

by GeneMapper software 3.0 (Applied Biosystems, Foster City, CA) using an internal lane sizing standard.

The summer and fall ancestry of the 2015 natural spawners and hatchery brood adult along with the 2016 out-migrating juveniles was evaluated using Structure 2.3.4 (Pritchard et al., 2000). Parentage analysis of the 2016 juveniles employed both Cervus 3.0.3 (Kalinowski et al., 2007) and Colony 2.0.4.1 (Jones and Wang, 2010). The genetic diversity of both adult and juvenile samples was estimated using the linkgage disequilrium method in NeEstimator 2.01.

4 RESULTS AND DISCUSSION

4.1 Brood Year 2016 Summer Chinook Adult DNA Sampling

For BY 2016 summer Chinook, a total of 429 DNA samples were collected, representing 54% of the total escapement (Table 2). However, only 172 of the 378 adults above the diversion dam, or 46%, were DNA sampled (Table 2), which is below the proportion sampled in 2015 (91%) and the overall target number of 50-75% of wild spawners above the dam. This will be valuable for identifying and assigning BY 2016 naturally-spawned Chinook fry to known parents that spawned naturally above the diversion dam.

Table 2. Brood year (BY) 2016 summer Chinook (SCK) escapement and DNA sampling summary.

Group	Number	# DNA samples
Broodstock & Hatchery removals	321	201
Transported to Comox Lake (natural spawners)	168	168
SCK above Diversion Dam (natural spawners)	210	4
SCK below Diversion Dam (natural spawners)	90	56
Total SCK Return	**789**	**429**
Proportion BY 2016 Total Return DNA sampled		**~54%**
Proportion BY 2016 natural spawners above the diversion dam that were DNA sampled		**~46%**

Approximately 128 of the 321 adults removed from the system were used as hatchery broodstock (Table 2) and both females and males were also screened for BKD. Results from the ELISA screening are summarized in Table 3. The three groups listed in Table 3 denote the three separate holding locations of the adults at Rosewall and Big

Qualicum Hatcheries, based on their migration timing and arrival at the Puntledge River hatchery. Group 1 are those adults that arrived between 21 May and 2 July; Group 2 are migrants from 8 – 31 July; and, Group 3 are migrants from 1-7 August. Group 1 were considered the "early" timing fish and were spawned together, while Groups 2 and 3 were considered the "late" timing fish and the majority were also spawned within their own groups.

Table 3. BY 2016 summer Chinook broodstock BKD screening summary for both females and males. Groups 1-3 represent separate adult holding groups based on their migration timing.

		2016 BKD Summary – Females and Males				
	Migration Timing	NEG + LLD	LP	MP	HP	Totals
Females	**Group 1**	0	5	6	0	11
	Group 2	3	3	0	0	6
	Group 3	7	16	2	0	25
	Grand Total	**10**	**24**	**8**	**0**	**42**
	Percent of Total	**23.8%**	**57.1%**	**19.0%**	**0.0%**	
Males	**Group 1**	6	15	5	1	27
	Group 2	7	7	0	0	14
	Group 3	25	20	0	0	45
	Grand Total	**38**	**42**	**5**	**1**	**86**
	Percent of Total	**44.2%**	**48.8%**	**5.8%**	**1.2%**	

* Negative and Low level of detection (LLD) have been combined because they are treated the same.

4.2 Microsatellite Analysis

4.2.1 Parentage analysis of 2015 and 2016 returning jacks

DNA extractions and genotyping of the 2015 summer hatchery brood, and 2014 and 2015 natural summer spawners have been completed in preparation for conducting parentage analysis of the 2015 (n=20) and 2016 (n=71) returning jacks (table). DNA extractions of the 2016 summer jacks are currently being undertaken. It is expected that there will be some summer jacks present in the fall jack collection and therefore these will also be extracted for DNA. Once complete, the jack samples will be tested for parentage assignment to the natural spawners and hatchery brood parents in our database.

Table 4. Returning 2015 and 2016 adipose clipped (marked) and unmarked jacks submitted for genetic analysis.

	2015	2016
Summer		
Marked summer	20	71
Fall		
Marked fall	127	100
Unmarked fall		529
Total	**147**	**700**

4.2.2 Analysis of 2016 naturally-spawned summer Chinook salmon juveniles and assignment to 2015 adults that spawned naturally

Samples and ancestry

Tissue samples from 211 SCN adults sampled from the natural environment in the 2015 'homing study' and 'truck and transport' operations and 702 naturally-spawned juveniles collected between February and July during outmigration in the spring of 2016 were successfully analyzed at 15 microsatellite loci (genetic markers) for parentage assignment and a 'sex ID' locus to determine the sex of the parental spawners and juvenile fish (Table 5). The 211 natural spawners were estimated to be approximately 59% of the summer Chinook natural spawners that emigrated past the hatchery brood collection point in the lower river during 2015 (Withler and Guimond 2016). One sample among the adult was determined to have been sampled twice and excluded from the genetic analysis. In total, 693 juveniles were retained for analysis after duplicates, non-Chinooks, and failed genotypes were excluded.

Table 5. Puntledge summer Chinook tissue samples submitted and retained for genetic analyses.

Collections	Tissues submitted (n)	DNA extractions (n)	Duplicated genotypes (n)	Non-Chinook or genotyping failed (n)	Retained for analysis (n)
2015 natural summer spawners	211	211	1	0	210
2016 juveniles	702	709	6	10	693

The assignment program used to assign juveniles to their parents provides assignments to 'known' parents for which genotypes have been provided and to 'unknown' unsampled 'phantom' parents for which genotypes are deduced from the progeny genotypes.

A low assignment rate of juveniles to naturally spawning summer parents prompted a family analysis using hatchery fall brood as parents and a Structure analysis was conducted to explore the summer/fall genetic nature of the juveniles and natural spawners. The Structure analysis revealed that 23.8% of the juveniles (n=165) were of fall ancestry (i.e. summer score ≤0.5; Figure 2a). Seventy-one of these were subsequently identified as the progeny of the 2015 hatchery fall brood juvenile releases from the light avoidance study (Guimond et al. 2016a). Only 9 of the 71 juveniles assigned to the hatchery fall brood were collected in Wolf trap/RST mixed vials (i.e. combined samples collected from the Puntledge diversion dam evaluation facility wolf traps and an in-stream rotary screw trap or RST). The majority of the hatchery fall juveniles from the light avoidance study were sampled at the evaluation facility.

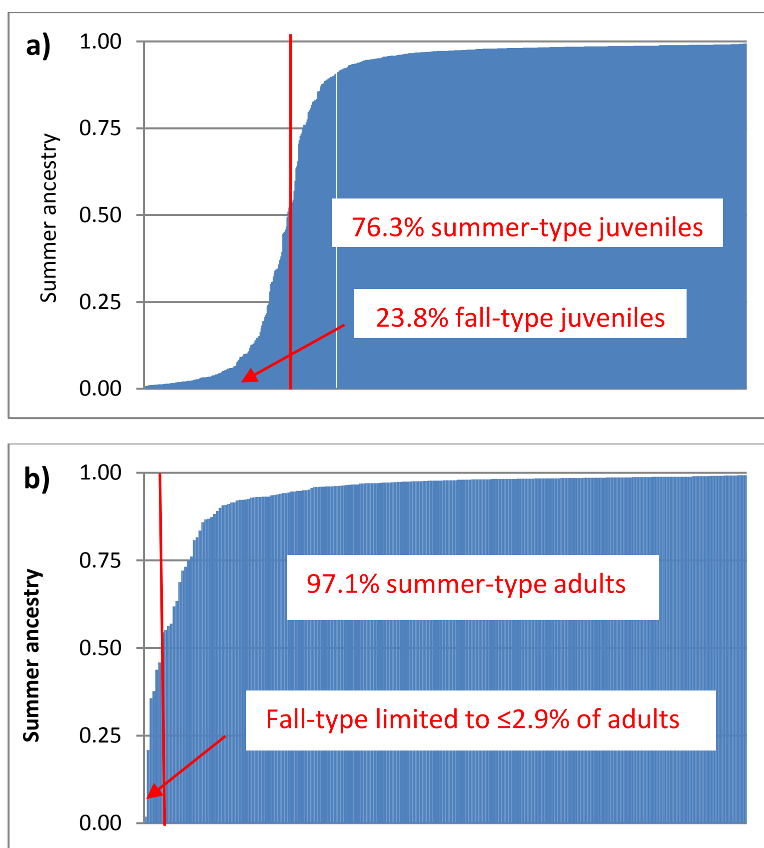

Figure 2. Structure inferred summer ancestry of 2016 juveniles (1a) and 2015 natural spawners (1b). The proportion summer ancestry attributed to each individual is given as a vertical blue bar.

Of the remaining 622 juveniles, 524 carried a summer genetic signature but 98 were clearly fall-like. These 98 falls (15.8% of the juvenile sample) were potentially the product of fall Chinook spawners above the diversion dam, in what is regarded as a

discrete summer Chinook spawning area. Their parents were not sampled. Since natural fall spawning is outside of the scope of this study, these fall juveniles were excluded from additional analyses. It is likely that fall Chinook salmon accessed the habitat above the diversion dam due to a late closure of the diversion dam fishway. Typically, Puntledge Hatchery closes the fishway in mid to late August to prevent early arriving fall Chinook passage into this habitat. In 2015, careful video surveillance of migration in the lower river, and regular observations of arrivals at the diversion dam delayed closure until mid-September in an attempt to allow as many summer Chinook as possible above the dam.

Family analysis: spawner success

The Structure analysis of natural summer spawners revealed that hatchery practices allowing early arriving adults access to the summer spawning grounds has been successful separating summer and fall spawners in so far as the majority of these adults (97.1%) were characterized by a strong genetic summer signal (Figure 2b). Two of the 6 fall-like adults were successful spawners. One spawned with a summer-like female and the other spawned with a phantom or unsampled female.

The majority (90.1%) of potential natural spawners were unmarked adults: there were 21 marked and 189 unmarked adults sampled in 2015 (Table 6). Not all hatchery releases are clipped therefore we expect some proportion of the unmarked adults are in fact hatchery origin. For brood years 2010 to 2013, the mark rate of the 0+ Chinook smolts released (percent that were adipose clipped and coded wire tagged) ranged between 24 and 43 %.

In the final year of the Homing Behaviour study (Guimond et al. 2016b), no spawning success data was available from PIT tagged and DNA sampled adults; none of the 8 PIT tagged adults reached the summer spawning grounds above the diversion dam (i.e. they we not detected by antennas at the dam).

Many of the natural spawners were either unsuccessful spawners or their progeny were not sampled; 92 of 202 adults transported to Comox Lake were not assigned as parents. However, 54.5% of transport adults were assigned progeny. Females were generally more successful that males; 67.6% of female produced 1 or more offspring whereas only 46.9% of males were successful spawners.

The limited number of marked adults in 2015 prevented a proper evaluation of marked and unmarked spawner success. Unmarked males (48.2%, n=114)) were generally more successful than marked males (33.3%, n=). When males and females were combined there was no difference in spawning success among marked and unmarked adults; both were 54-55% successful.

Table 6. Number and percentage of summer male and female adults sampled in 2015 that were assigned as parents to one or more of the 524 progeny sampled in the 2016 juvenile outmigration.

	Homing			Transport			Total		
	n	Assigned (n)	Assigned (%)	n	Assigned (n)	Assigned (%)	n	Assigned (n)	Assigned (%)
Females									
Mk*	0	0	NA	6	6	100.0	6	6	100.0
Nmk	3	0	0	68	44	64.7	71	44	62.0
Total	3	0	0	74	50	67.6	77	50	64.9
Males									
Mk	1	0	0	14	5	35.7	15	5	33.3
Nmk	4	0	0	114	55	48.2	118	55	46.6
Total	5	0	0	128	60	46.9	133	60	45.1
Total	8	0	0	202	110	54.5	210	110	52.4

* Not all hatchery releases are marked, thus 'Unmarked' adults may be hatchery origin or wild.

Missing parents

A low assignment rate of progeny to parents indicated that there were more unsampled spawners than was expected. Ninety percent (90%) of progeny were assigned to one or more sampled natural spawning adults. This was however, 9% below the expected given that 91% of the potential spawners present above the diversion dam were estimated to have been sampled (Table 7). Forty-four percent (44%) of progeny were not assigned or assigned to only a single parent. While 56% of progeny were assigned to 2 parents this was much lower than the expected assignment rate of 83%. There was little difference in the proportion of missing moms and dads, whereas in 2014 there were proportionately more moms missing.

Table 7. Expected and observed proportion and number of progeny (n=524) assigned to sampled natural spawning parents.

Progeny assigned to known parents	Probability of dam being sampled	Probability of sire being sampled	Expected proportion of progeny assigned	Expected progeny assigned (n)	Actual proportion	Actual progeny (n)
to both parents	0.91	0.91	0.83	434	0.56	295
to Dad, no Mom	0.09	0.91	0.08	43	0.15	79
to Mom, no Dad	0.91	0.09	0.08	43	0.18	95
to neither parent	0.09	0.09	0.01	4	0.10	55
Total			1.00	524	1.00	524

The proportion of progeny with phantom parents is probably the better estimate of the fraction of missing parents. It was estimated that we failed to sample roughly 25% of the natural spawners (

Table 8~~Table 8~~). It is probable that some adults were missed passing through the fishway during review of video surveillance files. The monitoring equipment experienced a prolonged period with limited nighttime visibility caused by electrical failure of the underwater lighting system.

Table 8. Number and percentage of known sampled and unsampled phantom males and females identified as parents in the summer fraction of juveniles (n=524). The percentage of progeny accounted for by each parental type is also shown.

	Parents		Progeny	
	(n)	(%)	(n)	(%)
Known dam	50	61.0	390	74.4
Phantom Dam	32	39.0	134	25.6
Total	82		524	
Known sire	60	58.3	374	71.4
Phantom Sire	43	41.7	150	28.6
Total	103		524	

Family Sizes

A skewed distribution of family size reflected the difficultly in collecting a random sample (Figure 3). Despite applying different juvenile sampling strategies (evaluation facility Wolf traps, rotary screw trap, and dip net) the aggregation of juveniles into families likely contributed to whole families being missed or poorly sampled. While families as large as 27-37 progeny were observed, 18-22% of families consisted of a single fish. The average family size was between 5.1 and 6.4 fish (Table 9~~Table 9~~).

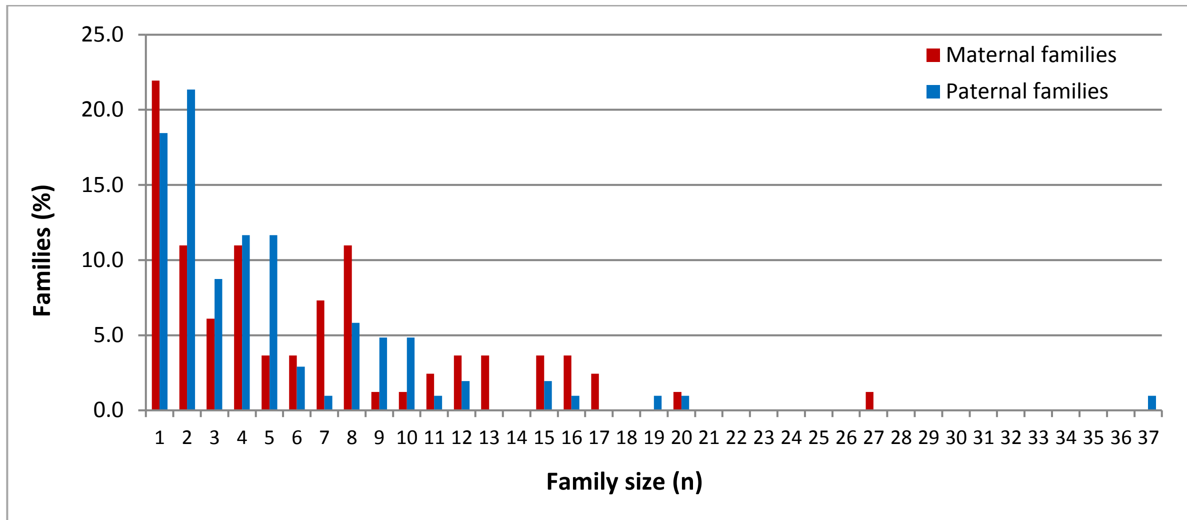

Figure 3. Maternal and paternal half-sib family sizes (BY15). The percentage of maternal or paternal families with a given number of progeny is given.

Table 9. Number of 2015 parents and family sizes (range and average) are given by sex.

	Parents	Family size		
	n	Minimum	Maximum	Mean
Dams	82	1	27	6.4
Sires	103	1	37	5.1

Polygamy

The overall sex ratio in the sampled natural spawners was 2:1 (male:female). Polygamy was slightly higher in females. Seventy-four percent 74.4% of females spawned with multiple partners, whereas 62.1% of males spawned with multiple partners (Figure 4). Females on average spawned with 3.1 partners; for males it was 2.5 (

Table 10~~Table 10~~).

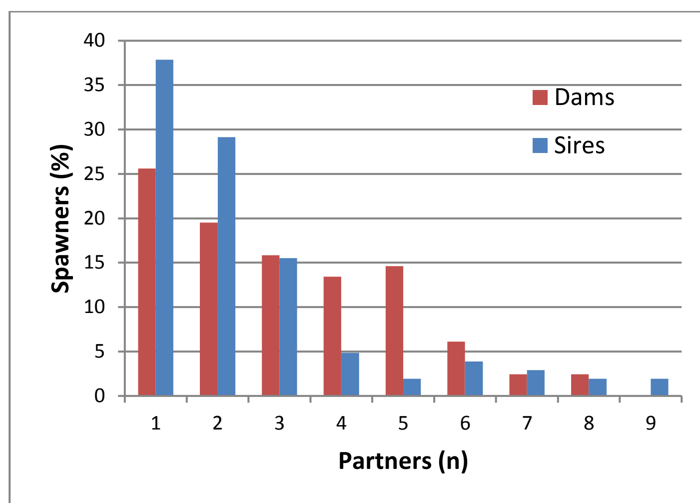

Figure 4. Polygamy of 2015 natural spawners. The proportion of dams or sires spawned with a given number of partners is given by sex.

Table 10. Polygamy in BY15. Percentage of polygamous adults and the number of partners (range and mean) are given for each sex.

			Polygamous partners		
	n	Polygamy (%)	Minimum (n)	Maximum (n)	Mean (n)
Dams	82	74.4	1	8	3.1
Sires	103	62.1	1	9	2.5

Effective population size (genetic diversity)

The 'genetic effective size' or N_e of a population can be estimated from the microsatellite genotypes. Factors that reduce the genetic effective size of a population from one generation to the next include:

1) a high proportion of unsuccessful potential parents

2) an unequal sex ratio (both sexes contribute half the genetic information to the next generation; if successful spawners of one sex are scarce, then half the genetic diversity of the next generation comes from only a few males or females

3) highly unequal contributions of successful parents to the next generation, again limiting the numbers of fish that contribute diversity to succeeding generations

The 'effective size' estimates for samples of the Puntledge SCN that were used as 2015 hatchery brood, those that constituted the potential natural spawners of 2015 and of the sampled natural progeny are shown in Figure 5 and Table 11. More genetic diversity

was observed in natural summer spawners compared with the hatchery summer brood. It should be noted that in describing the natural summer spawners, 6 adults with fall genetic ancestry (3% of the natural spawners) were excluded from diversity calculations.

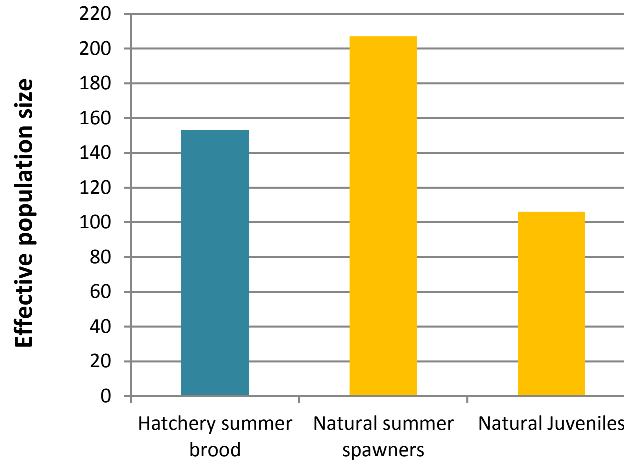

Figure 5. Effective population sizes (N_e) for the 2015 hatchery summer brood, 2015 sampled natural spawners, and 2016 out-migrating summer juveniles. The harmonic mean of the number of individuals sampled is shown at the top of each bar.

Table 11. Effective population sizes (N_e) for the 2015 hatchery summer brood, 2015 sampled natural spawners, and 2016 outmigrating summer juveniles. The harmonic mean of the number of individuals sampled and the 95% confidence limits are given.

	Harmonic mean n	N_e	95% Confidence Interval
Hatchery summer brood	155	153.3	138.8-170.5
Natural summer spawners	203	207.1	187.4-230.4
Natural Juveniles	507	106.2	102-110.6

Genetic diversity in the sampled spawners was not effectively transferred to the out-migrating juveniles: only 51.3% of spawner diversity seen in their progeny. Whether this is sampling effect or a real issue will be determined in the evaluation of returning jacks and adults, or through increased sampling rate/effort on the juvenile outmigrants.

A smaller 2015 summer brood collection was reflected in lower N_e brood estimate which was less than half that observed in 2014 (Withler and Guimond 2016). This difference was in proportion with the number of adults taken for brood; roughly 280 and 155 were taken in 2014 and 2015, respectively.

In contrast, the genetic diversity was higher in the 2015 natural spawners (n=210) despite nearly half as many natural spawners being sampled in 2015 compared with 2014 (n=373). Genetic diversity was generally higher in males than females both in the brood and male spawners. This was likely attributed to a more varied age distribution in the spawning males.

In the hatchery brood all the genetic diversity was captured in the males alone. Hatchery brood females did not add to the brood diversity. Natural spawning males and females captured different fractions of the total genetic diversity. The N_e estimate for males and females combined was at least 20% than for either sex alone. Genetic diversity was higher in males in both the hatchery brood and natural spawners. The lower diversity among females may be the result of family structure (Figure 6, **Error! Reference source not found.**~~Table 12~~).

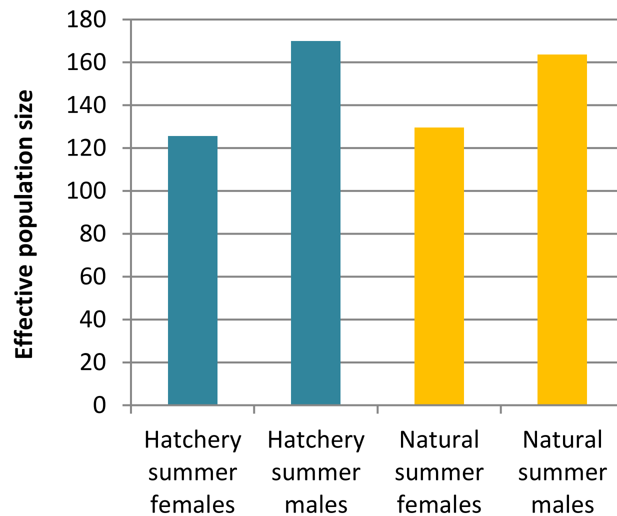

Figure 6. Effective population sizes (N_e) for the 2015 hatchery Summer brood and sampled natural Summer spawners given by sex. The harmonic mean of the number of individuals sampled is shown at the top of each bar.

Table 12. Effective population sizes (N_e) for the 2015 hatchery Summer brood and sampled natural Summer spawners given by sex. The harmonic mean of the number of individuals sampled is shown at the top of each bar.

	Harmonic mean n	N_e	95% CI
Hatchery summer females	86	126	110.4-144.8
Hatchery summer males	70	170	138.2-218.1
Natural summer females	74	130	110.6-154.9
Natural summer males	130	164	146.3-184.9

Genetic diversity and juvenile out-migration timing

There was temporal pattern to the distribution of genetic diversity among the out-migrating juveniles. Neither the early nor late emergent juveniles capture all of the juvenile genetic diversity (Figure 7, Table 13). Early juveniles had 69.1% of total juvenile diversity while more was captured in late fraction (80.6%).

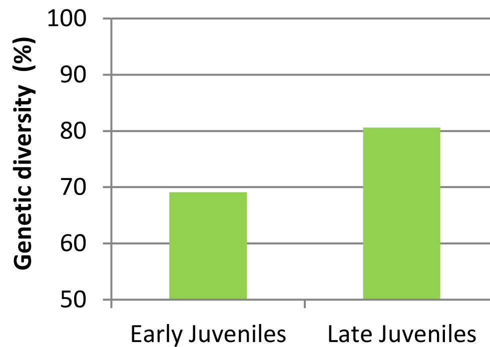

Figure 7. Genetic diversity of the early and late emergent 2016 juveniles. The genetic diversity (%) is given by the ratios of effective population sizes of the early or late juveniles to the entire juvenile sample. The harmonic mean of the number of individuals sampled is shown at the top of each bar.

Table 13. Effective population sizes (N_e) of 2016 out-migrating juveniles. 95% confidence interval (CI) and ample sizes (n and harmonic mean n) are given. Genetic diversity (N_e %) of the early and late juvenile fractions is given as a percentage of the total juvenile diversity.

	n	Harmonic mean n	N_e	95% CI	N_e (%)
All Juveniles	524	507	106	102-110.6	
Early Juveniles	222	212	73	69.8-77.2	69.1
Late Juveniles	302	295	86	81.5-89.8	80.6

This becomes significant when evaluating genetic diversity in the context of our current knowledge of juvenile summer Chinook migration and survival in the upper Puntledge River. Past studies have shown that the early emergent migration period may account for over 75% of the total juvenile out-migrating population, and that the small size of the emergent juveniles (i.e. 35-50 mm fork length) in this time period (Feb – April) puts them at greater risk of entrainment and mortality at the Hydro facility. Hydro-related mortality of juveniles with fork lengths less than 50 mm would result in a 19.4% loss of genetic diversity in 2015 alone. The cumulative effect of selective mortality to the early emergent juveniles is therefore a concern for the preservation of existing genetic diversity in summer run.

February was a critical month in the early emergence period; not only was there a surge in the number of out-migrating juveniles (n=155) but their contribution to genetic diversity was also greater than for the smaller March fraction (n=34; Figure 8, Table 14).

Mortality of the February out-migrating juveniles would result in a 12.6% loss in genetic diversity. This diversity was not captured in any of the other out-migration months. In contrast, the mortality of either the March, April or May juvenile out-migrators did not impact overall juvenile diversity to the same extent because the individuals from families in these fractions are also seen out-migrating in other months.

June and July fractions were also critical months in terms of their contribution the genetic diversity of the juveniles (i.e. diversity not observed in the balance of the juvenile sample). Juveniles in these later months carried 10.5% and 6.1% of the total diversity, respectively. Preservation of both early and late juvenile fractions is necessary to sustain existing diversity.

For 2015, there was no sampling of juveniles between March 2 and 24. A review of the 2014 data to evaluate the contribution made by March out-migrants to the total juvenile diversity found that mortality had no effect on N_e. However, this was based on a very small sample size: only 29 fry were sampled between 28 February and 30 March for BY 2014.

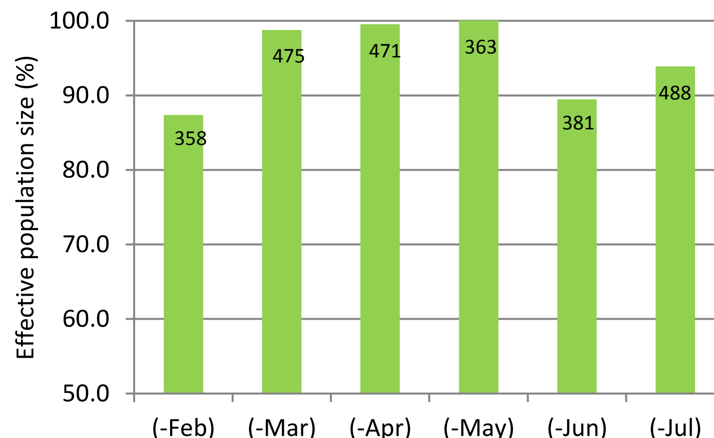

Figure 8. Genetic diversity in simulated juvenile mortality events. The genetic diversity (%) is given by the ratio of effective population size for juvenile fractions with select mortality to the entire juvenile sample. Mortalities were simulated by excluding out-migrating juveniles out-migrating in select months (eg. (–Feb) simulates the mortality of all February out-migrating juveniles). The harmonic mean of the number of individuals sampled is shown at the top of each bar.

Table 14. Simulated effective population sizes for out-migrating juveniles with given mortalities occurring in select months (eg. (–Feb) simulates mortality of all February out-migrating juveniles). Effective population size is given as a percentage of the total juvenile sample.

	n	Harmonic mean n (0.01)	N_e (0.01)	95% CI (0.01)	% N_e (0.01)	% lost genetic diversity
All	524	507	106	102-110.6		
All (-Feb)	369	358	93	88.7-97.1	87.4	12.6
All (-Mar)	490	475	105	100.6-109.3	98.8	1.2
All (-Apr)	486	471	106	101.4-110.2	99.5	0.5
All (-May)	375	363	113	107.8-118.5	106.4	0.0
All (-Jun)	396	381	95	90.9-99.4	89.5	10.5
All (-Jul)	504	488	100	95.8-103.8	93.9	6.1

Juvenile out-migration timing (family effects)

There were 222 early and 302 late emergent juveniles. Differences in family out-migration timing accounted for the non-random distribution of genetic diversity in the juvenile sample. Families differ in the terms of the composition of early and late emergent progeny; 20.7% of maternal families were exclusively early- and 36.6% exclusively late out-migrating (Table 15).

The variation in the family out-migration was statistically supported (Table 16). An ANOVA showed that there were significant differences in the mean out-migration time among half-sib families. Maternal half-sib families account for more of this variation than do paternal half-sib families (maternal F=11.9, paternal F=11.9, P<0.0001).

The loss of genetic diversity in this early out-migration period was in part due to the loss of entire families (**Error! Reference source not found.**~~Figure 9~~); 17 maternal half-sib families with juveniles smaller than the 50 mm fork length were observed solely in the early emergent period (before April 25).

Table 15. Out-migration timing in maternal half-sib families (BY15). Families are described by the run timing of the offspring within each family; "Mixed" families had both early and late out-migrating offspring.

Family type	Families (n)	Families (%)
Early	17	20.7
Mixed	35	42.7
Late	30	36.6
Total	82	

Table 16. ANOVA of out-migration timing in maternal and paternal half-sib families.

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between maternal half-sibs families	681799.2	63	10822.2	11.9	8.2181E-63	1.3
Within maternal half-sibs families	401770.3	442	909.0			
Total	1083569.5	505				
Between paternal half-sibs families	606739.2	83	7310.1	6.4	7.61594E-39	1.3
Within paternal half-sibs families	477346.5	421	1133.8			
Total	1084085.7	504				

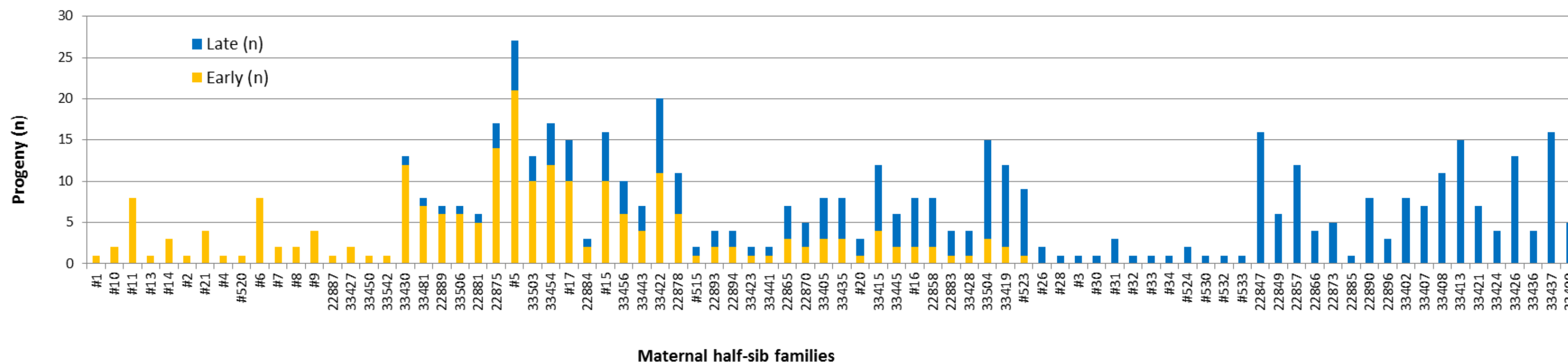

Figure 9. Out-migration timing in maternal half-sib families (BY15). The number of early and late out-migrating progeny in each family is given. The dam of each family is indicated; # indicates phantom dams.

5 CONCLUSIONS AND RECOMMENDATIONS

The following modifications to the 2017 Puntledge River Summer Chinook Salmon program are recommended to address some of the biological and sampling factors that may be responsible for our current observations of a low level of transmission of genetic diversity from parents to progeny in the naturally spawning component of the population:

1. Summer Chinook salmon adults transported to Comox Lake by hatchery staff will reflect the same sex ratio as the immigrating population captured at the lower hatchery (i.e. staff should not selectively remove females from the population to meet hatchery egg targets).
2. Puntledge Hatchery staff should increase DNA sampling on summer Chinook adults that bypassed the lower hatchery before they enter the headpond or Comox Lake. These adults represent a significant proportion of the early 'natural spawning' population, and 'phantom' parents. These missed fish can be captured and DNA sampled at the diversion dam fishway. This was piloted in 2016 and was very successful. Compared to conventional methods of seining the pool below the diversion dam, this procedure required fewer staff to implement, was less stressful on the fish and safer for crew.
3. Procedures for operating the diversion dam fishway to allow summer Chinook passage and restrict fall Chinook from habitat above the dam should be reviewed. Based on past DNA analysis of Puntledge summer Chinook, ~96% of adults arriving at the hatchery before August 1 are summer-type adults (Guimond and Withler 2010). However, this proportion declines throughout August with the summer-run representing less than 40% of arrivals by the third week of August. When required for brood, summer Chinook adults selected during this later timing period are confirmed through DNA analysis prior to spawning. This screening procedure has been effective in reducing the artificial production of summer-fall crosses at the hatchery. However, the likelihood of hybridization between the Puntledge summer and fall Chinook populations in the natural environment is high, and more difficult to control. Appropriate timing for closing the diversion dam fishway is critical to maximizing the natural spawning summer-run population above the dam and reducing the risk of fall Chinook access to this summer Chinook spawning refuge. DNA sampling of later migrating adults at the fishway (using methods in #2 above) will assist in verifying whether the closure date of the diversion dam fishway is sufficient for preventing early arriving fall Chinook from migrating above.

4. Increase the sampling rate on out-migrating summer Chinook juveniles captured at the diversion dam assessment facility over the entire migration period (Feb – July) from 10% to up to 20%. A low sampling rate results in the failure to detect the progeny of many parents. Considering that 18-22% of the families identified in the 2016 out-migrating juveniles were represented by a single offspring indicates that many low abundance families may have gone undetected at the current rate of sampling. In addition, a differentially higher mortality on the early emergent fry at the hydro facility reduces the capture rate of these early migrants and therefore may get missed by our sampling regime and artificially reduce measured family size.
5. Increase the sampling effort on out-migrating summer Chinook juveniles, particularly during the period when the Puntledge Generating Station is shutdown for maintenance, when the evaluation facility is not operating, and/or captures of fry at the facility are low or nil. Alternative trapping methods must be used to collect summer Chinook migrants at or upstream of the dam. It is possible that genetic diversity during this period largely overlaps with the earlier migration period, but goes undetected at our current low sampling rate. In addition, many of the apparently unsuccessful parents (no detected progeny) may have produced progeny that migrated primarily during the shutdown period.

6 ACKNOWLEDGEMENTS

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