Using Genetic and Phenotypic Comparisons to Evaluate Apparent Segregation among Kokanee Spawning Groups

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Abstract
Genetic and phenotypic traits of spatially and temporally segregated kokanee Oncorhynchus nerka spawning groups in Lake Pend Oreille, Idaho, were compared to test for evidence of divergence on the basis of ecotype (stream spawners versus shoreline spawners) and spawn timing and to describe morphological, life history, and reproductive variation within and among groups. Early and late spawning runs were found to be reproductively isolated; however, there was no clear evidence of genetic differentiation between ecotypes. Spawning groups within the same ecotype differed in length, age distribution, mean length at age, fecundity, and egg size. Variation in reproductive attributes was due primarily to differences in length distributions. Larger-bodied shore-spawning kokanee were located in areas where egg survival is known to be enhanced by downwelling, suggesting that the distribution of shore-spawning kokanee may be partly structured by competition for spawning habitats with groundwater influence. This study contributes to other research indicating that introduced kokanee populations are unlikely to undergo adaptive divergence if they have a history of population fluctuations and are supplemented regularly.

The Sockeye Salmon Oncorhynchus nerka and its nonanadromous form, kokanee, express the highest degree of spawning habitat plasticity of all Pacific salmon Oncorhynchus spp. (Foerster 1968; Burgner 1991). Oncorhynchus nerka is known to spawn in streams (Eiler et al. 1992; Garrett et al. 1998), backwater ponds (Hall and Wissmar 2004), lakeshore habitats (Kerns and Donaldson 1968), and even at depths greater than 20 m (Hassemer and Riemann 1981; Gipson and Hubert 1993). Systems that contain O. nerka can have single or multiple ecotypes, the most common of which are stream spawners and shoreline spawners.
are often sympatric, with temporally overlapping spawning runs (Blair et al. 1993; Quinn et al. 1999). *Oncorhynchus nerka* populations with both ecotypes and run timings are found in endemic populations (Taylor et al. 1997, 2000; Russello et al. 2012) and in human-established populations with multiple founding stocks (Burger et al. 2000). Surprisingly, however, reproductively isolated ecotypes within the same run are also found in human-established populations where a single founding ecotype has undergone adaptive divergence (Hendry et al. 2000; Hendry 2001; Lin et al. 2008). The most prominent example of rapid differentiation is the Sockeye Salmon population of Lake Washington, Washington, which was established using a single founding stock that was introduced between 1937 and 1945. This population diverged into reproductively isolated shore-spawning and stream-spawning ecotypes in 9–14 generations (Hendry et al. 1996, 2000; Hendry 2001). Phenotypic comparison studies and a crossing experiment involving the two ecotypes in Lake Washington identified significant differences in spawner body depth as well as distinct incubation temperature tolerances for stream- and shore-spawned embryos, suggesting that rapid differentiation was the result of local adaptation to particular spawning and incubation environments (Hendry et al. 1998).

Although rapid adaptive divergence of Lake Washington Sockeye Salmon is an important discovery, research in additional systems is needed to further understand the ecological conditions that foster differentiation of new stocks versus panmixia in human-established populations of *O. nerka*. Understanding the underlying stock structure of populations is important for managers because reproductive segregation can influence population viability (Ricker 1972; MacLean and Evans 1981). Multiple run timings and spatially isolated spawning may provide a buffer against die-offs in a particular habitat type and at a particular time of the year, thereby promoting long-term population stability (Hilborn et al. 2003). However, reproductive segregation could also exacerbate population declines if precise homing separates spawners and reduces per-capita reproductive rates (Frank and Brickman 2000; Ying et al. 2011).

For several reasons, Lake Pend Oreille, Idaho, is a valuable study area for examining reproductively segregated stocks of *O. nerka*. First, the lake contains several introduced kokanee stocks with both ecotypes and multiple run timings—an “early run” in late September and a “late run” in November–December. Second, the degree to which spawning groups (based on ecotype and run timing) are separated is relevant to how the kokanee population in the system is managed. Kokanee in Lake Pend Oreille provide a valuable recreational fishery and serve as a prey resource for recreationally valuable Rainbow Trout *Oncorhynchus mykiss* and native Bull Trout *Salvelinus confluentus* (Clarke et al. 2005; Hansen et al. 2010). Managers supplement stream-spawning ecotypes by stocking kokanee in one of the lake’s tributaries, and they manipulate the water level of Lake Pend Oreille to improve near-shore spawning habitat for the shore-spawning ecotype (Wahl et al. 2011). Stream spawners are thought to be primarily of hatchery origin, whereas shore-spawning fish are thought to be naturally produced. If ecotypes are found to be reproductively isolated, then managers can continue to focus on enhancing the ecotypes separately. Alternatively, if mating and habitat preferences are flexible, then managers can be assured that enhancement efforts in either environment will affect the entire population rather than a particular ecotype.

The goal of this study was to use genetic markers and phenotypic traits to understand the basis for apparent segregation of kokanee spawning groups in Lake Pend Oreille. Specific objectives for accomplishing this goal were to (1) assess whether there is evidence of adaptive divergence of kokanee based on ecotype or reproductive isolation according to run timing, (2) compare phenotypic traits among and within kokanee ecotypes, and (3) identify patterns among spawning groups that could be exploited to better manage kokanee in the system. Comparisons involved a total of nine spawning groups: three stream-spawning groups from the early run; two stream-spawning groups from the late run; and four shore-spawning groups, also from the late run (Table 1). Genetic analyses were carried out using species-specific microsatellites and single-nucleotide polymorphism (SNP) markers. Phenotypic traits that were evaluated included spawner length, age, origin (i.e., natural versus hatchery), fecundity, and egg mass.

**METHODS**

*Study area.*—Lake Pend Oreille is meso-oligotrophic, with a surface area of 38,000 ha, a mean depth of 164 m,

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shoreline</strong></td>
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<tr>
<td>Shoreline</td>
<td></td>
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<tr>
<td>Bernard Mine</td>
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<td>Evans Landing</td>
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<tr>
<td>Idlewilde Bay</td>
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<tr>
<td>Scenic Bay</td>
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<tr>
<td><strong>Stream</strong></td>
<td>Cabinet Gorge Hatchery</td>
<td>Granite Creek</td>
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<tr>
<td>Granite Creek</td>
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<tr>
<td>Trestle Creek</td>
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<tr>
<td>Granite Creek</td>
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<tr>
<td>South Gold Creek</td>
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</table>
and 310 km of shoreline (Figure 1). Most of the lake is deep (>100 m) and contains steep, rocky shorelines, with the exception of the north end. The morphometry of the lake creates ideal conditions for a pelagic fish assemblage, which was historically dominated by Cutthroat Trout Oncorhynchus clarkii, Bull Trout, and Pygmy Whitefish Prosopium coulterii and now includes introduced populations of kokanee, Lake Whitefish Coregonus clupeaformis, and Lake Trout Salvelinus namaycush. The outlet of Lake Pend Oreille is the Pend Oreille River, and the lake’s largest tributary is the Clark Fork River, which makes up 90% of the drainage. Additional tributaries include the Pack River and several smaller streams.

Kokanee in Lake Pend Oreille are derived from several sources. The species first entered the lake in the 1930s, when they emigrated from Flathead Lake, Montana, via the Clark Fork River. At that time, Flathead Lake contained a strain of kokanee that was endemic to Lake Whatcom, Washington (Waples et al. 2011). Prior to 1970, kokanee in Lake Pend Oreille were late-run fish, consisted of both ecotypes, and spawned throughout the lake (Jeppson 1955). Regular stocking of Lake Pend Oreille began in 1973 after a significant decline in the kokanee population and has continued until the present. Kokanee eggs are gathered each fall from females collected at a weir on Sullivan Springs Creek (a tributary of Granite Creek); fry are reared at the Cabinet Gorge Hatchery prior to release in the early summer. During the 1970s, an early spawning run was established in Trestle Creek by using kokanee from Deadwood Reservoir, Idaho, which are of an unknown origin (Waples et al. 1997). Additional early-run kokanee from Deadwood Reservoir and Kootenay Lake, British Columbia, were stocked in Trestle and Granite creeks during the 2000s (Wahl et al. 2011).

At the time of this study, shore-spawning aggregations in Lake Pend Oreille were located at Evans Landing, Bernard Mine, Idlewilde Bay, and Scenic Bay. Underwater camera surveys had recently identified spawning activity at depths down to 30 m in some areas. Evans Landing is a steep and bouldery shoreline on the western shore of the lake, whereas Bernard Mine is located beneath cliffs on the southern end of the lake. Idlewilde Bay has a moderate shoreline slope and is more exposed than Scenic Bay, which is shallower and not as steep and generally contains more fine sediment (Whitlock et al. 2014a). Scenic and Idlewilde bays are situated within the recharge area of the Spokane Valley–Rathdrum Prairie Aquifer (Hsieh et al. 2007). Groundwater influence in these areas, especially Scenic Bay, has been recently linked with increased survival of kokanee embryos (Whitlock et al. 2014a, 2014b).

Early-run kokanee consist of only the stream ecotype and primarily spawn in Granite Creek, North Gold Creek, South Gold Creek, Trestle Creek, and the Clark Fork River. Tributaries where late-run kokanee are known to spawn include Granite Creek, South Gold Creek, and several other streams. Kokanee that spawn in the Clark Fork River travel up to 15 km upstream to the fish ladder at the Cabinet Gorge Hatchery. The smaller tributaries, which are thought to contain the majority of kokanee (Granite, South Gold, and Trestle creeks), have roughly similar watershed areas (~5,000 m²) and habitat characteristics (Saffel and Scarnecchia 1995; USFWS 2002).

Field sampling.—Spawning kokanee were sampled on six separate occasions in four tributaries to Lake Pend Oreille, which included the Clark Fork River (Cabinet Gorge Hatchery), Granite Creek, South Gold Creek, and Trestle Creek (Table 2). Hereafter, fish sampled at the same location and during the same run (early or late) are referred to as a “spawning group.” Stream-spawning kokanee were sampled during September–November 2011 except those in Trestle Creek, which were sampled during September 2012. On most occasions, fish were collected from streams by using a Smith-Root LR-24 backpack electrofisher (Smith-Root, Vancouver, Washington). Electrofishing was extremely efficient because streams were less than 5.0 m wide and less than 0.2 m deep, and the majority of spawners did not move from redds. Early spawners from Cabinet Gorge Dam and late spawners from Granite Creek were collected by using dip nets rather than electrofishing. Fish were netted directly from the Cabinet

FIGURE 1. Map of the Lake Pend Oreille basin, Idaho, depicting the locations where kokanee spawning groups were sampled for genetic and phenotypic comparisons in 2011–2012.
TABLE 2. Sample sizes of kokanee collected from spawning groups across two runs (early and late) and among stream and shoreline areas in the Lake Pend Oreille basin, Idaho, 2011–2012. The total sample is the number of fish collected from each spawning group; the remaining columns describe the number of individual fish that were subsampled for comparisons involving fin rays, otoliths, genetic analyses, and eggs.

<table>
<thead>
<tr>
<th>Spawning group</th>
<th>Sample date(s)</th>
<th>Total sample</th>
<th>Fin rays</th>
<th>Otoliths</th>
<th>Genetic</th>
<th>Fecundity</th>
<th>Egg mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabinet Gorge Hatchery</td>
<td>Sep 22, 2011</td>
<td>75</td>
<td>65</td>
<td>48</td>
<td>50</td>
<td></td>
<td></td>
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<tr>
<td>(stream–early)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Granite Creek (stream–early)</td>
<td>Sep 20, 2011</td>
<td>260</td>
<td>73</td>
<td>53</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Gold Creek (stream–late)</td>
<td>Nov 9 and 23, 2011</td>
<td>408</td>
<td>71</td>
<td>53</td>
<td>56</td>
<td>125</td>
<td>95</td>
</tr>
<tr>
<td>Granite Creek (stream–late)</td>
<td>Nov 9 and 28, 2011</td>
<td>323</td>
<td>76</td>
<td>74</td>
<td>62</td>
<td>139</td>
<td>46</td>
</tr>
<tr>
<td>Bernard Mine (shore–late)</td>
<td>Nov 23 and 29, 2011</td>
<td>50</td>
<td>46</td>
<td>21</td>
<td>23</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Evans Landing (shore–late)</td>
<td>Nov 28 and 29, 2011</td>
<td>133</td>
<td>73</td>
<td>58</td>
<td>63</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Idlewilde Bay (shore–late)</td>
<td>Nov 23, 2011</td>
<td>153</td>
<td>76</td>
<td>45</td>
<td>37</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Scenic Bay (shore–late)</td>
<td>Oct 27, Nov 4, and Nov 8, 2011</td>
<td>514</td>
<td>86</td>
<td>81</td>
<td>48</td>
<td>174</td>
<td>26</td>
</tr>
<tr>
<td>Trestle Creek (stream–early)</td>
<td>Sep 23, 2012</td>
<td>77</td>
<td>47</td>
<td>47</td>
<td>63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*120 kokanee sampled on November 8, 2011, were only used in the fecundity comparison.

Gorge Hatchery fish ladder and from the egg-take weir on Sullivan Springs Creek.

Shore-spawning kokanee aggregations were sampled on seven occasions and at four shoreline locations, including Bernard Mine, Evans Landing, Idlewilde Bay, and Scenic Bay (Table 2). Shore-spawning aggregations were located with the aid of an echosounder and an underwater camera. Sinking experimental gill nets (3.0 m deep × 45.7 m long, containing graded mesh varying from 25.4- to 63.5-mm bar measure) were deployed in spawner aggregations. Gill nets were set for 30 min or less at depths between 10 and 50 m depending on the locations of the spawner aggregations. On November 4, 2011, kokanee in Scenic Bay were sampled by using floating gill nets (6.1 m deep × 91.4 m long, with 63.5-mm bar mesh) for the purpose of collecting egg samples. These fish were used in comparisons of egg size but were not included in any length comparisons due to the differences in gill-net mesh sizes that were used.

Overall, 1,993 kokanee were sampled across nine different spawning groups. Hard structures were collected from 607 kokanee, of which 475 were subsampled for genetic analyses (Table 2). The total number of kokanee collected among spawning groups varied from 50 to 514 individuals, and genetic sample sizes varied from 23 fish at Bernard Mine to 63 fish at Trestle Creek, Granite Creek, and Evans Landing. Spawning aggregations of kokanee were located and sampled at depths shallower than 20 m in Scenic Bay, between 10 and 40 m in Idlewilde Bay, and between 30 and 50 m at Bernard Mine and Evans Landing.

Genetic comparison.—Gill filament and opercle tissue was removed from the frozen heads of subsampled kokanee and preserved in 100% ethanol. After DNA extraction (nexttec Genomic DNA Isolation Kit; XpressBio, Thurmont, Maryland), each sample was amplified with 16 microsatellite loci: Omni1070 (Rexroad et al. 2001), One13 (Scribner et al. 1996), Oki1 (Smith et al. 1998), Ssa407, Ssa408 (Cairney et al. 2000), Oneu114, Oneu112, Oneu115, Oneu104, Oneu108, Oneu103, Oneu106, Oneu111, Oneu110 (Olsen et al. 2000), Otx103 (Beacham and Wood 1999), and Omy77 (Morris et al. 1996). The PCR conditions and thermocycler protocols are available from the corresponding author upon request. All PCR products were electrophoresed using an ABI 3730 automated sequencer (Applied Biosystems, Inc. [ABI], Foster City, California) following the protocols described by Ackerman et al. (2012). Chips were imaged on a Fluidigm EP1 system and were analyzed and scored using Fluidigm SNP Genotyping Analysis Software version 3.1.1. Resulting genotypes were stored on a Progeny database server housed at the Idaho Department of Fish and Game genetics laboratory. Genotypes for both marker sets were obtained from a total of 465 samples (Table 2). Microsatellite and SNP data sets were analyzed separately.

One additional SNP was also screened on all samples: One_68810 was previously identified as a significant outlier differentiating the shore-spawning and stream-spawning ecotypes in multiple river basins across the...
distribution of *O. nerka* (Nichols et al. 2016; Veale and Russello 2017a, 2017b). At this SNP, stream-spawning ecotypes predominantly exhibit the T allele, whereas shore-spawning ecotypes predominantly exhibit the G allele. Although several studies have conducted restriction-site-associated DNA sequencing, interrogating thousands of SNPs, no other SNP has exhibited this magnitude of differentiation across the two ecotypes (Veale and Russello 2017a). Genotyping of *One_68810* was accomplished using a recently developed TaqMan assay (*One_LRRC9_68810*) following the protocols described by Veale and Russello (2017b).

After genotyping was completed, genetic analyses were performed to describe the relative genetic diversity and relatedness of spawning groups. Each spawning group was tested for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium with GENEPOP (Raymond and Rousset 1995). A modified false discovery rate (Benjamini–Yekutieli FDR) procedure was used to adjust for multiple comparisons and to ensure that the experiment-wise α level was approximately equal to 0.05 (Narum 2006). Genetic diversity was measured by the number of alleles per locus (*N*ₐ), observed heterozygosity (*H*ₒ), and expected heterozygosity (*H*ₑ), which were calculated by using the Microsatellite Toolkit for Microsoft Excel (Park 2001). Exact G-tests were also performed to assess the significance of genetic differentiation between pairs of populations and to estimate pairwise values of the population differentiation index *F*ₜₛₚ (Weir and Cockerham 1984). To examine genetic relationships among populations, genetic distances (Cavalli-Sforza and Edwards 1967) between all populations were estimated via GENDIST in PHYLIP version 3.5 (Felsenstein 1993). A neighbor-joining dendrogram was generated from these genetic chord distances with the program FITCH in PHYLIP. Bootstrap replicates of 1,000 iterations were attained with SEQBOOT, and a consensus tree was formed with CONSENSE in PHYLIP. The dendrogram generated in PHYLIP was visualized using TREEVIEW version 1.6.6 (Page 1996). For the SNP data set, a discriminant analysis of principal components (DAPC) was also performed using the R package “Adegenet” (Jombart 2008) to visually depict genetic relationships between kokanee spawning groups.

Evidence of genetic differentiation among groups was further assessed by performing individual assignment tests using the program ONCOR (Anderson et al. 2008; Kalnowski et al. 2008). In addition to addressing a study objective, these tests also determined whether observed genetic differentiation among groups would be sufficient for conducting genetic stock identification (i.e., the practice of decomposing a mixed sample of animals into genetically distinct populations). The ability to perform genetic stock identification would mean, for instance, that if kokanee were sampled from a midwater trawl in Lake Pend Oreille and genotyped, then the contribution of each spawning group to the population could be estimated. Individual assignment tests were performed on both the microsatellite data set and the SNP data set.

**Phenotypic trait comparison.**—Length information was collected from all fish that were sampled, fin rays and otoliths were collected from a subsample of fish within each group, and fecundity and egg size were compared among several groups (Table 2). Body length was measured as the mid-eye-to-fork length to avoid bias in length comparisons resulting from fin deterioration and heterogeneity in secondary sexual characteristics (Burgner 1991; Burger et al. 1995). Up to 10 fish per centimeter length-group were subsampled for aging and otolith examination. Kokanee were aged by examining both the leading pectoral fin rays and the sagittal otoliths (Bilton and Jenkins 1969). Fin rays were removed at the base, mounted in centrifuge tubes with epoxy, and sectioned using a Buehler Isomet low-speed saw (Buehler, Lake Bluff, Illinois) to facilitate viewing with a stereoscope (Koch and Quist 2007).

Otoliths were also examined to determine whether kokanee were of hatchery or wild origin. All kokanee reared at the Cabinet Gorge Hatchery are thermally marked by fluctuating the daily temperature that fry experience immediately after hatching (Volk et al. 1999). Alternating temperatures produce a unique “barcode” pattern on fish otoliths that can be identified microscopically. Thermal marks were identified by mounting the sagittae (sulcus-down) on microscope slides using thermoplatic cement and polishing both sides with 100-μm wet–dry sandpaper and 0.3-μm alumina slurry (Marshall and Parker 1982; Stevenson and Campana 1992; Wetzel 1993).

Length and age distributions were compared between aggregations from the same ecotype to ensure that differences were not influenced by gear selectivity. Pairwise Kolmogorov–Smirnov (K–S) tests with a Bonferroni correction were used to identify significant differences in the shape and position of length distributions (Neumann and Allen 2007). Analysis of variance was also used to compare mean lengths of spawning groups within each ecotype (Zar 2009). Ages were assigned to all fish by constructing age–length keys using the subsample of aged fish. Separate age–length keys were created for each spawning group by using the semirandom age–length key approach described by Iserman and Knight (2005). The relationship between length and age was also investigated by estimating mean length at age for each spawning group based on the assigned ages for all fish that were sampled. The probability that an individual spawner was of hatchery origin was modeled using logistic regression (Hosmer et al. 2013) with length, age, and spawning group as explanatory variables. One logistic regression model contained age, spawning group, and an interaction, and
another model contained length, spawning group, and an interaction. Likelihood ratio tests were used to test for the interaction in either model and to test for significant effects of predictors.

Eggs and ovaries were sampled from late-spawning kokanee across several sites to measure fecundity and egg mass (Table 2). Fecundity provides an indicator of the per-capita reproductive rate, whereas egg mass reflects individual investment in offspring and has been positively related to embryo survival under favorable incubation conditions (West and Larkin 1987; Murray et al. 1989). Egg size was further relevant to the objectives of this study because this trait is subject to selection pressure based on the habitat conditions in the incubation environment (e.g., substrate size and temperature; Sargent et al. 1987; Blair et al. 1993; Quinn et al. 1995). Fecundity samples were only collected when eggs were not flowing or could not be made to flow from the vent by compressing the body cavity. Egg size samples were collected from females whose eggs were gravid and either flowing from the vent readily or loose within the body cavity (Kinnison et al. 1998). After sampling, all kokanee eggs were preserved in a solution of buffered 4% formalin to accurately preserve their mass (Fleming and Ng 1987). Fecundity was estimated by subsampling ovaries (or egg samples) by weight. Samples were divided in half by weight; eggs on one of the halves were counted, and the number of eggs was then multiplied by 2 to obtain a fecundity estimate. Egg size was estimated by sampling approximately 30 eggs from each female and weighing each egg individually. Reproductive characteristics were compared among breeding groups by adjusting for length using a linear model (i.e., ANCOVA; Zar 2009). A general linear model was used to compare fecundities; a linear mixed-effects model estimated using maximum likelihood was used to compare egg sizes (Zuur et al. 2009). The linear mixed-effects model included a random intercept, which accounted for the correlation in egg size measurements within individual fish. Likelihood ratio tests were used to examine for interactions and main effects in the egg size model.

#### RESULTS

**Genetic Comparison**

**Microsatellite analysis.**—The $H_E$ per locus was very similar across the nine spawning groups, varying from 0.86 to 0.88 (Table 3). The average number of alleles

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**Table 3.** Summary table describing microsatellite and single-nucleotide polymorphism (SNP) data obtained from kokanee spawning groups that were sampled in the Lake Pend Oreille basin, Idaho, 2011–2012. Spawning group, sample size ($N$), expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), number of alleles per locus ($N_A$), and the number of loci that deviated from Hardy–Weinberg equilibrium (HWE) expectations are presented. The number of private alleles ($P_A$) is shown for the microsatellite analysis. The frequencies (%) of the G and T alleles observed at SNP One_68810 are also shown.

<table>
<thead>
<tr>
<th>Spawning group</th>
<th>$N$</th>
<th>$H_E$</th>
<th>$H_O$</th>
<th>$N_A$</th>
<th>HWE</th>
<th>$P_A$</th>
<th>One_68810 G allele</th>
<th>One_68810 T allele</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microsatellites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cabinet Gorge Hatchery (stream–early)</td>
<td>50</td>
<td>0.88</td>
<td>0.87</td>
<td>17.6</td>
<td>0/16</td>
<td>7</td>
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<tr>
<td>Granite Creek (stream–early)</td>
<td>63</td>
<td>0.87</td>
<td>0.85</td>
<td>18.1</td>
<td>1/16</td>
<td>9</td>
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<tr>
<td>Trestle Creek (stream–early)</td>
<td>63</td>
<td>0.86</td>
<td>0.87</td>
<td>17.9</td>
<td>3/16</td>
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<td>Granite Creek (stream–late)</td>
<td>62</td>
<td>0.86</td>
<td>0.86</td>
<td>16.8</td>
<td>0/16</td>
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<td>South Gold Creek (stream–late)</td>
<td>56</td>
<td>0.88</td>
<td>0.89</td>
<td>17.2</td>
<td>0/16</td>
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<tr>
<td>Bernard Mine (shore–late)</td>
<td>23</td>
<td>0.88</td>
<td>0.85</td>
<td>12.8</td>
<td>0/16</td>
<td>1</td>
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<tr>
<td>Evans Landing (shore–late)</td>
<td>63</td>
<td>0.87</td>
<td>0.86</td>
<td>17.1</td>
<td>0/16</td>
<td>5</td>
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<tr>
<td>Idlewilde Bay (shore–late)</td>
<td>37</td>
<td>0.87</td>
<td>0.88</td>
<td>15.3</td>
<td>0/16</td>
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<td>Scenic Bay (shore–late)</td>
<td>48</td>
<td>0.86</td>
<td>0.82</td>
<td>15.3</td>
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TABLE 4. Pairwise comparisons of the genetic differentiation index $F_{ST}$ based on microsatellite loci (above the diagonal) and single-nucleotide polymorphism loci (below the diagonal) from nine kokanee spawning groups sampled from stream and shoreline (shore) locations during early and late spawning runs in the Lake Pend Oreille basin, Idaho, 2011–2012. Comparisons in bold italics exhibited significant genic differentiation ($G$-test).

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<td>0.007</td>
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<td>0.008</td>
<td>0.007</td>
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<td>0.005</td>
<td>0.006</td>
<td>0.005</td>
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<td>0.007</td>
<td>0.004</td>
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<tr>
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<td>0.007</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.004</td>
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<tr>
<td>South Gold Creek (stream–late)</td>
<td>0.005</td>
<td>0.007</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.004</td>
<td>0.024</td>
<td>0.022</td>
</tr>
</tbody>
</table>
observed per group varied from 12.8 (Bernard Mine–late) to 18.1 (Granite Creek–early). The highest number of private alleles observed in a spawning group was 12 from Trestle Creek–early (Table 3). All private alleles were observed at frequencies less than 5%. There was no evidence that any of the loci deviated more from HWE or linkage expectations than would be expected by chance \((P = 0.009)\). One spawning group, Trestle Creek–early, showed departures from HWE at 3 of the 16 loci. However, there was no consistent pattern of excess or deficiency.

Tests of genetic differentiation among sites, as measured by \(F_{ST}\) estimates and tests of genic differentiation, indicated low differentiation among groups with the same run timing (Table 4). Among the three early spawning groups, \(F_{ST}\) estimates varied from 0.005 to 0.008. None of the pairwise genic tests among these three spawning groups was significant \((P < 0.05)\). Similarly, little genetic differentiation was observed among late-spawning groups. Pairwise \(F_{ST}\) varied from 0.004 to 0.009, and no pairwise tests of genic differentiation were significant (Table 4). High levels of genetic differentiation were observed in comparisons among early and late-spawning groups; pairwise \(F_{ST}\) varied from 0.018 to 0.034, and all genic differentiation tests were significant \((P < 0.05)\). This pattern of genetic population structuring observed in tests of genetic differentiation was apparent within the neighbor-joining dendrogram, which clustered early spawning groups together with 100% bootstrap support (Figure 2). Within the late-spawning groups, the geographically proximate groups from Scenic Bay, Bernard Mine, and Idlewilde Bay clustered together with 83.3% bootstrap support.

Individual assignment tests in ONCOR showed low accuracy in correctly assigning individuals back to spawning groups (Table 5). All spawning groups except the Trestle Creek group (67.3%) exhibited accuracies less than 50%. However, when spawning groups were consolidated into reporting groups by run timing, the assignment tests were much more accurate (Table 5). For all spawning groups, greater than 90% of the samples were assigned back to the correct reporting group. Six of the nine spawning groups exhibited 100% accuracy.

**Single-nucleotide polymorphism analysis.**— Tests of genetic differentiation among sites by using the SNP marker set showed patterns similar to those observed with the microsatellite marker set. The \(H_F\) per locus was similar across the nine spawning groups, varying from 0.25 to 0.27 (Table 3). No spawning groups showed significant departures from HWE, and there was no evidence that any of the loci deviated more from HWE or linkage expectations than would be expected by chance \((P < 0.05)\). Among the three early spawning groups, \(F_{ST}\) estimates varied from 0.004 to 0.008 (Table 4). None of the pairwise genic tests among these three groups was significant. Low genetic differentiation was observed among late-spawning groups; pairwise \(F_{ST}\) varied from 0.004 to 0.011, and none of the pairwise tests of genic differentiation was significant (Table 4). Similar to the microsatellite data set, higher levels of genetic differentiation were observed in comparisons among early and late-spawning groups. However, pairwise \(F_{ST}\) values \((0.076–0.095)\) were roughly three times higher than those observed with the microsatellite data set. These higher differentiation values were largely driven by several loci that exhibited large allele frequency differences among early and late-spawning groups (Table 6). For example, locus *One_rab1a-76-A1* exhibited an allele (T) that was present at a frequency of 63–70% in early spawning groups but was absent from all late-spawning groups except South Gold Creek, where it was present at a frequency of 1%.

The neighbor-joining dendrogram based on genetic distances calculated from the SNP data set showed a topology very similar to that produced from microsatellites. Early spawning groups clustered together with 100% bootstrap support (Figure 2). Within the late-spawning groups, Scenic Bay, Idlewilde Bay, Evans Landing, and Bernard Mine clustered together with 71.2% bootstrap support.
The DAPC also showed distinct clustering of early spawning and late-spawning populations (Figure 3). The Trestle Creek–early samples formed a broad but somewhat distinct group from the two other early spawning groups (Cabinet Gorge Hatchery–early and Granite Creek–early).

Individual assignment tests indicated that the SNP data set did not yield any higher accuracy in assigning individuals to their correct spawning group (Table 5). However, SNPs did yield slightly higher assignment accuracies when spawning groups were combined according to run timing (early versus late). For all sites, 100% of the samples were assigned back to the correct reporting group; the exception was Cabinet Gorge Hatchery, for which 96.9% of samples were correctly assigned (Table 5). Screening of the One_68810 SNP, which had yielded nearly fixed allelic differences between shore- and stream-spawning ecotypes in previous studies, generated discordant results (Table 3). As expected, all shore-spawning sample collections were fixed or nearly fixed for the G allele; however, the G allele was also the dominant allele observed in two of the late stream-spawning collections: Granite Creek–late (96.7%) and South Gold Creek–late (93.8%).

**Phenotypic Comparisons**

Models used to compare phenotypic traits met assumptions based on the examination of residual plots and goodness-of-fit tests (e.g., global F-test, likelihood ratio). One of the spawning groups in the logistic regression model (Trestle Creek–early) contained 0% hatchery fish, which caused a complete separation of points that prevented the accurate estimation of SEs. To address this issue, Trestle Creek was omitted from the model. Logistic regression models for

<table>
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<th>Spawning group</th>
<th>N</th>
<th>% correct</th>
<th>Largest misidentification</th>
<th>N</th>
<th>% correct</th>
<th>Largest misidentification</th>
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The table describes the proportion of baseline individuals that were correctly assigned using microsatellite markers and single-nucleotide polymorphism loci (SNPs). The proportion of correct assignments using spawning group as the reporting group is shown in the “Spawning Group Assignment” section; the proportion of correct assignments using run timing as the reporting group is shown in the “Run Assignment” section. The number of samples (N) and percentage of correct assignments (% Correct) are described. The spawning group to which baseline samples were most commonly misclassified (“Largest misidentification”) is also provided. Samples with missing data at any locus were removed from analyses.
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<th>Locus</th>
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<th>Granite Creek (stream–early)</th>
<th>Trestle Creek (stream–early)</th>
<th>Granite Creek (stream–late)</th>
<th>South Gold Creek (stream–late)</th>
<th>Bernard Mine (shore–late)</th>
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spawner origin were not overdispersed based on the dispersion parameter, and showed adequate fit based on the area under the receiving operator characteristic curve (>0.7).

Length distributions varied in both ecotypes. Early stream-spawning kokanee had greater variability in length than late stream spawners (Figure 4). The Trestle Creek–early spawning group’s length distribution and mean length estimate were significantly smaller than those of the other two early groups (Cabinet Gorge Hatchery–early and Granite Creek–early; \( P < 0.01 \)). Length distributions of the two late stream-spawning groups (Granite Creek–late and South Gold Creek–late) also differed significantly based on K–S tests (\( P < 0.05 \)). However, the mean lengths of these two late-spawning groups did not differ significantly (\( F_{1,729} = 3.0, P = 0.084 \)), suggesting that the difference was likely due to the greater variability in length distribution of the South Gold Creek spawning group. Pairwise K–S tests indicated that the length distribution of kokanee in Scenic Bay was significantly greater than the distributions in the other three shoreline locations (\( P < 0.05 \)). The other groups were not significantly different from one another. The mean length of kokanee in Scenic Bay was 259.87 ± 0.72 mm (mean ± SE), whereas the lengths of spawners at Bernard Mine, Evans Landing, and Idlewild Bay were 246.12 ± 2.02 mm, 242.17 ± 1.24 mm, and 243.47 ± 1.16 mm, respectively.

Age distributions of spawning groups varied from ages 2 to 4, but the majority of spawners were either age 3 or age 4, whereas only 3.6% of sampled fish were assigned to age 2 (Figure 5). Age distributions of early spawning groups were shifted lower relative to late-spawning groups. Except for the Scenic Bay spawning group, shore-spawning groups had an approximately even division between age-3 and age-4 fish. The Scenic Bay spawning group was unique in that the proportion of age-4 fish was more than quadruple the proportion of age-3 fish. Mean length-at-age estimates indicated that the Scenic Bay spawning group was also significantly greater in length at a given age than any other shore-spawning group (Figure 6).

Examination of otoliths indicated that hatchery-origin kokanee were present in all spawning groups except the Trestle Creek–early group, which was composed entirely
of wild-origin fish. The proportion of hatchery fish was influenced by spawning group but not by individual fish length ($\chi^2 = 0.530$, df = 1, $P = 0.466$) or age ($\chi^2 = 0.695$, df = 1, $P = 0.405$). Because neither length nor age significantly influenced the probability that a fish was of hatchery origin, a logistic regression model containing only the “spawning group” variable was interpreted. The other two early run groups were primarily of wild origin, with 22.6% (95% confidence interval [CI] = 9.0–46.1%) hatchery-origin fish sampled from Granite Creek–early and 16.7% (95% CI = 5.8–38.2%) hatchery-origin fish obtained from Cabinet Gorge Hatchery–early. Among late-spawning kokanee, a larger proportion of hatchery-origin fish spawned in streams than in shoreline areas. South Gold Creek–late was 54.7% (95% CI = 30.4–77.1%) hatchery origin, and Granite Creek–late was 97.3% (95% CI = 89.2–99.6%) hatchery origin. The hatchery component in shore-spawning groups varied considerably: 47.6% (95% CI = 27.5–68.3%) for Bernard Mine, 13.8% (95% CI = 4.7–32.9%) for Evans Landing, 22% (95% CI = 8.4–46.3%) for Idlewilde Bay, and 30.9% (95% CI = 14.3–54.5%) for Scenic Bay.

Mean fecundity and egg size varied among spawning groups; however, most of the variation was attributable to differences in kokanee length. The ANCOVA for fecundity indicated that the relationship between fecundity and length did not vary according to spawning group ($F_{2, 433} = 0.4; P = 0.685$). Fecundity was positively related to length ($F_{1, 435} = 112.6$, $P < 0.001$), and fecundity at length differed significantly among groups ($F_{1, 435} = 18.5$, $P < 0.001$). After accounting for the effect of length, post hoc Tukey tests revealed that fecundity of kokanee in the Scenic Bay spawning group was 12.5% (35.98 ± 9.64 [mean difference ± SE]; $P < 0.001$) greater than that of the Granite Creek–late spawning group and 23.8% (62.28 ± 10.40; $P < 0.001$) greater than that of the Idlewilde Bay spawning group. No significant interaction was identified between spawning group and length ($\chi^2 = 8.56$, df = 5, $P = 0.13$), suggesting that the relationship between length and egg size did not vary among groups. Egg size...
was positively associated with spawner length ($\chi^2 = 12.6$, df = 1, $P < 0.001$), but egg size at length did not differ among groups ($\chi^2 = 3.51$, df = 5, $P = 0.62$).

**DISCUSSION**

The goal of this research was to determine whether there was evidence of genetic differentiation and adaptive divergence among spatially and temporally segregated kokanee spawning groups in an intensely managed system. Genetic analyses suggested that shore-spawning kokanee were genetically more similar to one another than they were to stream-spawning kokanee with the same run timing (late run); however, the analyses did not reveal significant differentiation on the basis of ecotype (i.e., shore spawners versus stream spawners). This pattern was observed within the microsatellite and 93-SNP marker sets as well as in the allele frequencies at the SNP One_68810, which had been previously shown to exhibit divergence between these ecotypes over much of the species’ distribution in the Columbia, Fraser, and Snake River drainages (Nichols et al. 2016; Veale and Russello 2017a, 2017b). Our results did not show evidence for adaptive divergence of kokanee in Lake Pend Oreille, but significant temporal isolation was discovered among early and late spawning runs. Phenotypic trait comparisons revealed variation both among and within ecotypes. Length, age at maturity, fecundity, and egg size differed among spawning groups, similar to the findings of other studies (Killick and Clemens 1963; Woodey 1965; Averett and Espinosa 1968; Burger et al. 1997); however, variation in reproductive characteristics was largely due to differences in length distributions. Spatial separation, the absence of genetic structure, and differences in length and age composition among groups suggest that the spawning distribution of kokanee in Lake Pend Oreille is more likely structured by assortive mating than by natal homing.

Previous research provided reason to expect adaptive differentiation among kokanee spawning groups in Lake Pend Oreille based on the period for which kokanee have been present in the lake, their apparent spatial isolation in distinct habitats, and the known homing and spawning behaviors of *O. nerka*. Hendry and Kinnison (1999) described divergence of *O. nerka* occurring within several decades, and Lake Pend Oreille has contained multiple ecotypes of kokanee for more than 80 years. Spatial

![FIGURE 5. Age (years) as a percentage of catch among kokanee sampled from stream and shoreline locations during the early and late spawning runs in the Lake Pend Oreille basin, Idaho, 2011–2012. Ages were assigned by using age–length keys constructed from a subsample of fish in each group, for which age was estimated based on analyses of pectoral fin rays and otoliths.](image-url)
segregation between spawning groups in Lake Pend Oreille was also consistent with examples of reproductively isolated *O. nerka* spawning groups described in other studies (Hendry et al. 1998; Hendry 2001; Russello et al. 2012). The distance separating spawning groups in this study was 2–40 km, which is comparable to the distances between reproductively isolated Sockeye Salmon spawning groups in Tustumena Lake, Alaska (Burger et al. 1997). The distinctiveness of spawning environments in Lake Pend Oreille is another reason why divergence was considered a possibility. Shore-spawning kokanee, particularly those that spawn at great depths, deposit their eggs in a way that differs substantially from egg deposition by stream spawners: shore spawners often have poorly defined redds and have even been observed to broadcast eggs over boulder substrates (Kerns and Donaldson 1968; Hassemer and Rieman 1981). Underwater camera surveys in Lake Pend Oreille have documented shore-spawning kokanee depositing their eggs in small patches of gravel located between and on the ledges of boulder piles as well as on unstable gravel slopes below rockslide areas (Whitlock et al. 2014a). Kokanee in Scenic Bay spawn in large groups over beds of cobble and gravel that have been swept clean of fine sediment by multiple spawners. The final reason for suspecting that kokanee in Lake Pend Oreille might have differentiated is the species’ well-documented natal homing ability and spawning ground behavior. *Oncorhynchus nerka* home more precisely than any other salmonid (Altukhov and Salmenkova 1994) and have been known to demonstrate considerable site fidelity with regard to stream and shoreline locations (Burger et al. 1997; Quinn et al. 1999; Stewart et al. 2003). Hendry et al. (1995) found that Sockeye Salmon aggregations separated by less than 15 m strayed at a rate of only 3% in Iliamna Lake, Alaska. Furthermore, *O. nerka* have low straying rates relative to other species and have even been shown to select against strays from other groups (Hendry 2004; Lin et al. 2008).

Factors that likely contributed to the mixture of late-run kokanee in Lake Pend Oreille are previous population bottlenecks and ongoing stocking. Mixing among spawning groups is more likely to occur when population sizes are low or variable (Adkison 1995). Assuming that kokanee were able to reproductively segregate soon after entering Lake Pend Oreille, several population declines in the lake’s history could have counteracted reproductive isolation and brought about the current mixed state. For example, the kokanee population declined significantly in

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**FIGURE 6.** Mean length-at-age estimates (±SE) for kokanee spawning groups sampled from stream (upper panels) and shoreline (lower panels) locations during two spawning runs (early and late) in the Lake Pend Oreille basin, Idaho, 2011–2012. Ages (years) were assigned using age–length keys constructed from a subsample of fish in each group, for which age was estimated based on analyses of pectoral fin rays and otoliths.
the late 1960s and again more recently after the expansion of Lake Trout (Paragamian and Bowles 1995; Hansen et al. 2010). Continual stocking also likely contributed to admixture of kokanee, as was the case for kokanee stocked in Japanese reservoirs (Yamamoto et al. 2011). Hatchery-origin fish are more likely to stray (Quinn 1993), which means that annual stocking in Lake Pend Oreille likely undermined local adaptation. Local adaptation could also have been hindered by stocking because hatchery rearing is known to release some traits related to incubation from selection pressures (Reisenbichler and Rubin 1995; Heath et al. 2003). There are several cases in which O. nerka stocks have remained discrete despite new introductions (Burger et al. 1997; Young et al. 2004). However, the stocking events in those cases occurred occasionally or decades in the past. In addition, the fish that were released in those systems made up only a small percentage of the population, which is not the case in Lake Pend Oreille, where hatchery-origin fish typically comprise a large portion (16–60%) of the population (Paragamian and Bowles 1995; Wahl et al. 2011).

Although no evidence of differentiation on the basis of ecotype was found in this study, there was clear evidence of differentiation between spawning groups with different run timings, a pattern that can be exploited by managers. Temporal separation has been shown to be a stronger isolating force than spatial separation in other systems and with other salmonid species (Altukhov and Salmenkova 1994; Adkison 1995).

The phenotypic comparisons in this study produced findings that are relevant to management—most notably the differences in length distribution between spawning groups and the positive relationship between length and reproductive attributes. Early stream-spawning kokanee sampled in Granite Creek were, on average, greater in length than late stream-spawning groups. This pattern may imply that early-run kokanee in Lake Pend Oreille grow faster because of differential diet, behavior, habitat use, or food conversion efficiency, owing to their specific genetics. Length differences between groups are germane to management because length was positively related to fecundity, meaning that larger individuals could contribute more to recruitment. The top fecundity model in the analysis estimated that the fecundity of relatively large (270-mm) females was 45% (95% CI = 38–68%) greater than that of relatively small (220-mm) females. If managers are interested in maximizing natural fry production, then stream and shoreline environments with larger individuals should be given priority for habitat enhancements.

Phenotypic comparisons also revealed that kokanee in Scenic Bay tended to be larger, regardless of age, which is especially interesting when placed in the context of contemporary research on the incubation success of shore-spawning kokanee in Lake Pend Oreille. Whitlock et al. (2014b) found that kokanee preferred to spawn in ground-water-influenced areas of Scenic and Idlewilde bays and that embryos in those areas had substantially higher survival than those in any other shoreline area, including Bernard Mine and Evans Landing. Segregation according to size is common among O. nerka (Foote 1988) and may be indicative of size-related competition for higher-quality habitat (Foote 1990; Quinn and Foote 1994). If differences in spawner length distribution imply size-related competition, then perhaps competition for downwelling habitat plays a role in structuring the spawning distribution of kokanee in Lake Pend Oreille. A similar pattern was reported for shore-spawning Brook Trout Salvelinus fontinalis in Canadian Shield lakes by Curry and Noakes (1995), who found that spawners competed for ground-water-influenced habitat on the shoreline of lakes.

There is a substantial body of literature on the adaptive divergence of O. nerka, but few studies have investigated these patterns in intensely managed fisheries. Findings from Lake Pend Oreille suggest that reproductive isolation among ecotypes may not be common in kokanee sport fisheries due to the influence of annual stocking programs. Although adaptive divergence was not detected in Lake Pend Oreille, the combination of genetic and phenotypic comparisons provided valuable insights that are useful to managers and contribute to the current understanding of kokanee spawning behaviors in lentic systems. As a result of this research, the reproductive isolation and size differences between early and late spawning runs in Lake Pend Oreille can be exploited to better achieve management goals. The clustering of spawners in distinct, spatially isolated environments further supported the claim that O. nerka have highly plastic spawning habitat requirements.

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