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Journal:	<i>Canadian Journal of Fisheries and Aquatic Sciences</i>
Manuscript ID	cjfas-2016-0065.R1
Manuscript Type:	Article
Date Submitted by the Author:	29-Jul-2016
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Keyword:	REPRODUCTION < General, ENDOCRINOLOGY < General, ANADROMOUS SPECIES < Organisms, Iteroparity, Skipped Spawning

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**Reproductive development in captive reconditioned female steelhead kelts:
evidence for consecutive and skip spawning life histories**

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Running title: Reproductive development in steelhead kelts

Keywords:

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Reconditioning of post-spawned anadromous rainbow trout (steelhead kelts, *Oncorhynchus mykiss*) is being implemented as a recovery tool on the Yakima River in the mid-Columbia River Basin. We assessed reproductive development in female Yakima River kelts by measuring plasma estradiol-17 β (E2) and vitellogenin (VG) levels during reconditioning in 2009-2011. Plasma E2 and VG levels showed fish separated into rematuring (consecutive spawning) and non-rematuring (presumed skip spawning) cohorts by October. Rematuration rates varied from 25% to 65%. Rematuring fish were consistently detected migrating toward spawning areas after release, whereas non-rematuring fish were occasionally detected on spawning migrations the following year. Rematuring fish grew more rapidly than non-rematuring fish over the reconditioning period and had higher muscle lipid levels and condition factor in October. Plasma E2 was elevated in rematuring fish by June to July, whereas plasma VG was elevated by June to August, suggesting that maturation decisions occur early in reconditioning. Rematuring and non-rematuring females could be separated by plasma E2 and VG levels by August to September, enabling separate management of consecutive and presumed skip spawners.

31 Introduction

32 After spawning, seasonally breeding iteroparous fishes may spawn again as consecutive
33 spawners (1 year spawning interval), or defer spawning for a year or more to spawn as skip
34 spawners (2 or more year spawning interval, Rideout et al. 2005, Jorgensen et al. 2006, Rideout
35 and Tomkiewicz 2011). The prevalence of skipped spawning may be higher than had been
36 previously appreciated, substantially reducing estimates of spawning biomass (Rideout et al.
37 2005, Rideout and Tomkiewicz 2011). Skipped spawning has been explained as an adaptive life
38 history decision driven by allocation of energy reserves under stochastic environmental
39 conditions (Jorgensen et al. 2006). If energy reserves are low, curtailing energy investment into
40 gonadal development during the year after spawning may decrease mortality, and the feeding
41 opportunities afforded by an additional year may result in increased growth and energy reserves,
42 increased competitive ability, and increased fecundity in female fish, offsetting the cost of
43 mortality over the additional year. While numerous field and modeling studies on skipped
44 spawning have been published in recent years, few studies have investigated the physiological
45 sequence of events that lead to the divergence of consecutive and skip spawning life histories in
46 post-spawning fishes (Burton 1994, Caldwell et al. 2013, Bangs and Nagler 2014, Caldwell et al.
47 2014).

48 Post-spawning anadromous rainbow trout (steelhead kelts, *Oncorhynchus mykiss*) provide
49 a model for the study of consecutive and skipped spawning. The extensive migration and
50 prolonged freshwater holding of stream-maturing (summer run) steelhead in large river systems
51 such as the Columbia River leads to an extreme energy deficit and high post-spawning mortality
52 (Penney and Moffitt 2014a, b, 2015). However, the fish that do repeat spawn express both the

consecutive and skip spawning life histories, and the proportion of skip spawners increases with increasing migration distance, consistent with regulation by energy reserves (Keefer et al. 2008).

The critical period hypothesis of salmonid maturation holds that the physiological decision to mature occurs during seasonally defined critical developmental windows (Campbell et al. 2006, Thorpe 2007, Sloat et al. 2014). During a window approximately one year prior to spawning, gonadal maturation is initiated if energy reserves are sufficient. During a second permissive window four to six months later, maturation is either continued or arrested, based on energy reserves. Energy reserves may be assessed in terms of body size, growth rate, and adiposity, and thresholds may vary based on sex, genetic background, age, and previous developmental history. However, the relative roles of these factors and the signaling pathways involved are not yet clear. While the critical period hypothesis was developed to explain initial maturation (puberty, e.g. Taranger et al. 2010), it is reasonable to assume that similar mechanisms are involved in rematuration in iteroparous species. Nonetheless, how these mechanisms interact with the extreme energy deficit incurred by migration and spawning in steelhead kelts is not known. In particular, it is unclear whether the functionally important decision window is the initiation or continuation window in repeat spawners. Given that the initiation window coincides with maximal energy depletion in kelts, it would be surprising if energy reserves at this time determined life history trajectory. Therefore, we hypothesize that all fish initiate rematuration, and the continuation window actually determines consecutive or skip spawning trajectory.

The reproductive endocrine axis regulates reproductive development in female salmonids. This physiological system consists of the brain, pituitary gland, ovary, and liver (Pankhurst 2008, Lubzens et al. 2010, Taranger et al. 2010, Wootton and Smith 2015). During maturation

76 decisions, the brain integrates environmental signals such as photoperiod and internal signals
77 related to energy reserves (Taranger et al. 2010, Wootton and Smith 2015). If conditions are
78 favorable, this results in neuroendocrine stimulation of pituitary gonadotropin secretion.
79 Circulating gonadotropins, particularly follicle stimulating hormone, stimulate ovarian
80 production of the principal female steroid, estradiol-17 β (E2). E2 in turn stimulates the liver to
81 produce the vitellogenins (VG). These large glycolipoproteins are taken up from the blood by
82 the developing oocytes and processed into the egg yolk. During maturation, plasma levels of E2
83 and VG increase by two to six or more orders of magnitude in rainbow trout (e.g. Bon et al.
84 1997, Wilkinson et al. 2010). Therefore, measurement of plasma E2 and VG levels from non-
85 lethal blood samples can be used to determine maturation status and track reproductive
86 development in female steelhead kelts.

87 In the Yakima River in the mid-Columbia Basin, captive steelhead kelt reconditioning is
88 being implemented as a recovery tool for populations listed under the Endangered Species Act
89 (ESA, Branstetter et al. 2011, Hatch et al. 2013, Trammell et al. 2016). The goal of the project is
90 to increase natural steelhead production by increasing the number of repeat spawners.
91 Downstream migrating kelts are collected during the spring at an irrigation diversion at Prosser,
92 Washington, on the lower Yakima River. Fish are treated for diseases and parasites, put into
93 tanks, and fed. Surviving kelts are released into the river in mid-October, with the intention that
94 these fish will migrate upriver to spawn. Kelt collections and releases are dominated by female
95 fish (93%, Hatch et al. 2013). However, the reproductive status of female Prosser kelts at release
96 in October has not been investigated. We hypothesize that consecutive and skip spawning
97 individuals are present, and that rematuration as a consecutive spawner is associated with
98 positive energy balance. If this is correct, then knowledge of the reproductive status of kelts at

the time of release would enable management of consecutive and skip spawners so as to maximize the contribution of both life history types to target populations.

The overall goal of this study was to characterize reproductive development during reconditioning in female steelhead kelts in the Prosser reconditioning project. Since these fish are ESA listed, we used non-lethal blood sampling and measurement of plasma E2 and VG levels to achieve this goal. Our specific objectives were to determine whether both rematuring (consecutive spawning) and non-rematuring (presumed skip spawning) individuals were present at the time of release, to investigate how maturation status affected migration patterns after release, to assess relationships between maturation status and growth and energy reserves during reconditioning, to begin to delineate when in the reconditioning process reproductive decisions were made, and to determine whether and when fish could be screened for maturation status based on plasma E2 and VG levels from a pre-release blood sample.

Materials and methods

Study Site

The Yakima River Basin is located in the mid-Columbia River Basin on the east slopes of the Cascade Range (Fig. 1). Prosser Diversion Dam (PD) is an irrigation diversion dam located on the lower Yakima River, below the spawning areas for the major Yakima River steelhead subpopulations (Satus Creek, Toppenish Creek, the Naches River, and the Upper Yakima River, Hockersmith et al. 1995, Frederiksen et al. 2015). Three fish ladders equipped with PIT tag detectors provide upstream passage for adult fish at PD. Water and a proportion of downstream migrating fish are diverted into the Chandler irrigation canal at PD, which is equipped with a fish bypass and collection system, the Chandler Juvenile Monitoring Facility (CJMF). Prosser Hatchery is located adjacent to the CJMF, approximately 500 meters downstream from PD.

122 *Kelt Collection and Reconditioning*

123 Yakima River steelhead kelts were collected and reconditioned at Prosser Hatchery (as
124 previously described in Branstetter et al. 2011, Hatch et al. 2013, Trammell et al. 2016). Briefly,
125 kelts were removed from the CJMF separator, anesthetized (tricaine methanesulfonate, 0.6 g l⁻¹,
126 buffered with NaHCO₃ to pH 7.0), measured for fork length, weighed, and tagged (PIT tag
127 inserted into the pelvic girdle). Fish selected for long term reconditioning were administered
128 oxytetracycline by intraperitoneal injection (20 mg kg⁻¹ body weight) to treat bacterial infections,
129 and ivermectin by gavage (0.4 mg kg⁻¹ body weight) to treat infestation with parasitic gill
130 copepods (*Salmincola californiensis*). Fish were held in four large circular tanks (6.1 m diameter
131 x 1.2 m height, flow rate 570 to 950 L min⁻¹) and four small circular tanks (3.0 m diameter x 1.2
132 m height, flow rate 140 to 240 L min⁻¹). Fish were held under natural photoperiod in well water
133 (13.8 °C), and were fed to satiation 3-5 times daily, initially with krill (parboiled frozen
134 *Euphausia superba*, Atlantic Pacific Products Inc., Kingston, RI) for one month and then with a
135 mixture of krill and pellets (BioBrood, 6.0 mm, BioOregon Inc., Longview, WA). Formalin
136 (37% formaldehyde, 1:6000 dilution, 1 hour) was administered five days per week to reduce
137 external infection with water mold (*Saprolegnia* spp.). Survival from spring collection to release
138 in the fall was 27.6% in 2009, 38.7% in 2010, and 32.8% in 2011 (Hatch et al. 2013).

139 *Sampling*

140 The length, weight, and sex were recorded for each fish at initial collection for
141 reconditioning and at release (10/29/2009, 10/13/2010, and 10/13/2011). In 2009 – 2011, female
142 fish were blood sampled at release. Release samples were a sub-sample in 2009 and 2010 (71 of
143 128 and 97 of 381 female fish, respectively), whereas in 2011 almost all fish were successfully
144 blood sampled (208 of 212 female fish). In 2009, one large tank of fish was blood sampled at

collection and release. In addition, a random sample of fish was taken from this tank for blood draws on 6/22/09 (30 fish) and 8/6/09 (31 fish). In 2010, one small tank of fish was serially sampled (collection, 7/21/10, 8/18/10, release 10/13/10), and in 2011, one large tank of fish was serially sampled (collection, 7/19/11, 9/8/11, release 10/13/11). Blood (2 ml) samples were drawn from the caudal vein using heparinized syringes (ammonium heparin, 10 mg ml⁻¹) and centrifuged (5 min, 5000 g) to separate cells from plasma. Plasma was collected and frozen on dry ice in the field prior to storage at -80°C. In addition to blood sampling, the PIT tag code, length, weight and sex of fish were recorded, and a reading of muscle lipid levels was taken with a microwave energy meter (Fish Fatmeter model 692, Distell Inc., West Lothian, UK), using the rainbow trout muscle lipid setting (Trout-1) at the two most anterior measurement sites recommended by the manufacturer (Crossin and Hinch 2005), as previously validated for measurement of rainbow trout muscle lipid levels (Caldwell et al. 2013).

Estradiol and Vitellogenin Assays

Plasma VG concentrations were assayed using a rainbow trout vitellogenin enzyme-linked immunosorbent assay (ELISA) kit (Biosense, Cayman Chemical, Ann Arbor, MI). Plasma samples were appropriately diluted and assayed in duplicate in the ELISA according to the manufacturer's instruction manual provided with the kit. Assay validation for *O. mykiss* plasma samples was conducted by the manufacturer. Intra-assay %CV was 6.9% and inter-assay %CV was 13.8%. Plasma E2 concentrations were assayed by radioimmunoassay (RIA) using commercially available kits (Coat-A-Count Estradiol (high range) and Coat-A-Count Estradiol Double Antibody (low range), Diagnostic Products, Los Angeles, CA) at the Center for Reproductive Biology Assay Core Laboratory (Department of Animal Sciences, Washington State University, Pullman, WA). Plasma samples were solvent extracted twice with diethyl ether

before use in the RIA protocol, and values were corrected for extraction efficiency (Caldwell et al. 2014). These assays and the extraction procedure have been validated for *O. mykiss* and closely related species (Campbell et al. 2006, Nagler et al. 2012, Caldwell et al. 2014). Intra-assay %CV was 3.4% and 6.6%, and inter-assay %CV was 6.3% and 9.0%, in the high and low range RIAs, respectively. Instead of assigning a zero value, plasma samples below the detection limit of the low range E2 assay (ED 80) or the VG assay (lowest standard used in the standard curve) were assigned a value of one half of the lowest detectable level (E2 2009: 0 samples, 2010: 5, 2011: 10; VG 2009: 0, 2010: 9, 2011: 6).

Post-Release Detections

Reconditioned kelts were released into the Yakima River at Prosser Hatchery. The Columbia Basin PIT Tag Information System (PTAGIS, www.ptagis.org) database was queried with a list of PIT tags of released kelts for detections of fish after release. Fish detected at PD or upstream sites from release until June 30th of the following spring were classified as migrating as consecutive spawners (*i.e.* migration pattern consistent with spawning the spring following release). Fish detected at tributary sites outside of the Yakima basin during this period were classified as consecutive spawning out of basin strays. Fish detected from July 1st of the following spring onward at any of the adult ladders at dams on the Columbia River downstream from PD were classified as migrating as skip spawners (*i.e.* migration pattern consistent with spawning two or more springs following release).

Data Analysis

Analysis was restricted to female fish positively identified by PIT tags that survived reconditioning. Plasma E2 and VG data were log₁₀ transformed prior to analysis. Fish were categorized into two groups at release based on cluster analysis of release plasma E2 and VG

191 levels. Cluster analysis was conducted using Ward linkage, data were standardized, and two
192 clusters were specified. Differences between post-release migration patterns of released
193 rematuring and non-rematuring fish, and the concordance between maturation status assigned by
194 necropsy versus by plasma E2 and VG levels, were assessed by Fisher's Exact (Chi-Squared)
195 test. Only consecutive spawning detections were included in this analysis; the number of skip
196 spawning detections was not sufficient for the test. The effects of maturation status, year, and
197 maturation x year on metrics of growth and energy reserves were assessed using a generalized
198 linear model (GLM, identity link function, normal distribution) due to unequal sample sizes.
199 Significant results were further analyzed by 1-way ANOVA and Tukey's HSD test. Potential
200 tank effects were tested first by one-way ANOVA to reduce the number of interaction terms.
201 Specific growth rate in length (SGRL, % change in length per day) was calculated as
202 $100 \cdot \ln(L_2/L_1)/\text{days}$, where L_2 and L_1 are release and collection lengths, respectively, and days is
203 the duration of reconditioning. Specific growth rate in weight (SGRW, % change in weight per
204 day) was calculated in a parallel manner using release and collection weights. Fulton's condition
205 factor (K) was calculated as $100 \cdot \text{weight (g)}/\text{length (cm)}^3$. Both muscle lipid levels measured
206 with the microwave energy meter and K were analyzed as metrics of muscle lipid levels and total
207 body lipid levels, respectively (Sutton et al. 2000, Caldwell et al. 2013). Correlations between
208 metrics of growth, energy reserves and plasma E2 and VG levels were estimated using restricted
209 maximum likelihood. The effects of maturation status, sampling date, and maturation x date on
210 plasma E2 and VG levels in fish sampled during reconditioning was assessed using a GLM
211 (identity link function, normal distribution), again due to unequal sample sizes. Collection data
212 were pooled over the range of dates indicated. The three years of data were analyzed separately
213 due to differences in sampling schedules and collection dates for experimental fish between

years. Significant results were further analyzed by 1-way ANOVA and Tukey's HSD test. Data are reported in the text as mean $\pm$ standard error. Statistical analyses were conducted using JMP 12.0.1 (SAS Institute, Cary, NC).

Results

Cluster analysis of VG and E2 values revealed division of female kelts into two groups at the time of release in October in 2009-2011 (Fig. 2). Threshold values of 0.1 mg ml⁻¹ VG and 500 pg ml⁻¹ E2 separated the two groups at the time of release. Fish in the group with high E2 and VG levels were classified as rematuring, and fish in the group with low E2 and VG levels were classified as non-rematuring. Due to insufficient plasma for both assays, a few additional fish were classified based on threshold values of either E2 or VG alone (2009: 5 fish, 2010: 8 fish, 2011: 0 fish). The percentage of rematuring female fish at release was 65% in 2009, 25% in 2010, and 54% in 2011. Rematuring female VG levels at release were $6.5 \pm .5$, 9.3 ± 1.1 , and $1.7 \pm .2$ mg ml⁻¹ in 2009-2011, respectively, whereas E2 levels at release were 66100 ± 5600 , 12600 ± 1600 , and 8700 ± 700 pg ml⁻¹.

Six mortalities occurred after the 10/13/2010 blood sampling during recovery from anesthesia prior to release. Necropsy of these mortalities showed that three were females with rematuring ovaries containing large follicles, two were females with non-rematuring ovaries, and one was a male with no evidence of maturation in the testis (Fig. 3). Seven mortalities occurred after the 10/13/2011 blood sampling during recovery from anesthesia. These were fish from a serially sampled tank that had been previously classified as rematuring females for a separate experiment, and all seven of these fish had rematuring ovaries with large follicles when necropsied. Combining years, all of the 10 females classified as rematuring based on release sample blood E2 and VG levels had rematuring ovaries with large follicles, and none of the two

females classified as non-rematuring based on blood E2 and VG levels had ovaries with large follicles, supporting the use of blood E2 and VG levels to determine maturation status (Fisher's exact test, $p = 0.0152$).

Maturation status at release influenced the post-release migration patterns of the fish, as inferred from detection at PIT tag arrays at PD and elsewhere (Table 1). Rematuring fish were detected migrating toward spawning areas during a time frame consistent with consecutive spawning at significantly higher rates than non-rematuring fish (Fisher's exact test, $p < 0.001$). In 2010, most fish known to be rematuring at release were kept for an additional experiment, resulting in a low sample size. However, approximately one quarter of the fish were blood sampled at release in 2010, and many more rematuring females were presumably released. All of the rematuring fish that were detected migrating as consecutive spawners were detected at locations at or upstream of PD, except one fish from the 2011 release that was detected at the Oasis Road Bridge on the lower Walla Walla River, giving an estimated out of basin straying rate of 1.5%. Fish from the non-rematuring release groups were detected migrating upriver during a time frame consistent with skip spawning, more than 8 months after release into the Yakima River. Skip spawners were detected returning at a low rate (2.9% overall), and the number of detections was not sufficient for contingency table analysis. Some non-rematuring fish were detected after release at Juvenile Bypass Systems at dams on the Columbia River below the Yakima River confluence during the fall (release date to Dec 31, 8 fish total) and spring (Feb 17 to May 12, 9 fish total) following release, suggesting downstream migration during these periods. Only a small proportion of downstream migrants would be expected to pass through these Juvenile Bypass Systems (Colotelo et al. 2014); therefore, the number of non-maturing fish

migrating downstream is probably higher. None of the detected downstream migrants were among the fish detected returning as skip spawners.

GLM analysis revealed that maturation status significantly affected all metrics of growth and energy reserves in reconditioned female kelts (Table 2). In contrast, the effects of year and the maturation x year interaction were only occasionally significant. No significant tank effects were found for growth and energy metrics. Rematuring female kelts grew more rapidly in both length and weight during reconditioning than non-rematuring fish, and had higher muscle lipid levels and K at release (Fig. 4). The effect of maturation status is analyzed and displayed separated by year for parallelism of presentation, illustrating the consistent pattern in spite of the loss of statistical power caused by separating years. Combining years, rematuring fish increased in length by 1.70 ± 0.12 cm and in weight by 0.84 ± 0.04 kg, whereas non-rematuring fish decreased in length by 0.43 ± 0.13 cm and increased in weight by 0.14 ± 0.04 kg over the course of reconditioning. Overall, SGRL and muscle lipid levels were lower in 2010 than in 2009 and 2011, and among non-rematuring fish, SGRW was higher in 2010 than in 2011. Within both rematuring and non-rematuring groups, growth metrics were correlated with each other (Table 3). Muscle lipid levels measured with the microwave energy meter and K were positively correlated, but the R^2 value was only 0.28 and 0.24 in rematuring and non-rematuring kelts, respectively. Plasma E2 and VG levels were positively correlated in both rematuring and non-rematuring female kelts. Interestingly, plasma E2 and VG levels were positively correlated with metrics of growth and energy reserves in non-rematuring fish. However, in rematuring fish, plasma VG level was not significantly correlated with any growth metric, and plasma E2 was only weakly correlated with SGRL, SGRW and K.

Reproductive development in rematuring and non-rematuring female kelts was tracked in serially sampled fish. In a GLM analysis, plasma E2 and VG levels were significantly influenced by maturation status at release, time of sampling, and the interaction of these factors (Table 4). Since significant interaction effects were found, the effects of maturation status and sampling time were assessed independently (Fig. 5). Plasma E2 and VG levels did not differ at collection between fish that subsequently rematured and fish that did not, except in 2009 when rematuring fish had significantly elevated E2 at collection. However, by the first sampling in June to July, E2 was significantly elevated in rematuring versus non-rematuring fish in all three years, and VG was elevated in rematuring fish in two out of three years. Rematuring fish maintained significantly elevated plasma E2 and VG levels over non-rematuring fish through the rest of the experiment.

Trends over time differed between E2 and VG, and between rematuring and non-rematuring fish. In rematuring fish, plasma E2 levels increased 438-fold to 1205-fold from collection to release, with significant elevations found by the first or second sampling point. In non-rematuring fish, plasma E2 levels also increased significantly over time in two of the three years, however, the increase was only 2.2-fold to 15.9-fold, and occurred later. In contrast, plasma VG levels in non-rematuring fish decreased 362-fold to 2157-fold from collection to release, becoming significant by the first sampling in two of three years. However, in rematuring fish, plasma VG levels increased only 7.2-fold to 23.5-fold from collection to release, with significant elevations not found until the second sampling point. In rematuring fish in 2010, plasma VG level did not change significantly over time, except for a decrease at the first sampling point. Overall, E2 increased strongly over time in rematuring fish but remained low in

non-rematuring fish, whereas VG decreased strongly over time in non-rematuring fish but remained high in rematuring fish.

Discussion

The major result of this study is that rematuring and non-rematuring reconditioned female kelts were present at the time of release in October, suggesting that both the consecutive and skip spawning life histories are expressed by reconditioned kelts. This finding is based on the division of fish into two groups, one with high plasma E2 and VG levels and another with lower levels. Elevations in plasma E2 and VG are established as indicators of reproductive maturation in female salmonids and other fishes (Pankhurst 2008, Lubzens et al. 2010, Taranger et al. 2010, Wootton and Smith 2015). Further support for classification of maturation status by plasma E2 and VG levels was provided by necropsy of post-sampling mortalities, and by detection of a significantly larger proportion of rematuring kelts migrating toward spawning areas after release versus non-rematuring kelts. The presence of the two life-history types had not been previously recognized in steelhead kelt reconditioning. Rematuration was strongly associated with positive energy balance during reconditioning, consistent with expectations based on the energetic cost of ovarian development and spawning migration (Thorpe 2007, Keefer et al. 2008, Sloat et al. 2014). More surprisingly, plasma E2 and VG levels diverged quite early between rematuring and non-rematuring fish, suggesting that maturation decisions are made early in the reconditioning process. This refutes our initial hypothesis that the functional decision point might occur later, but is consistent with models of the regulation of initial maturation in salmonids (Campbell et al. 2006, Thorpe 2007, Taranger et al. 2010, Kendall et al. 2015). Maturation status of female kelts could be determined from plasma E2 and VG levels from mid-August onward, enabling separate management of consecutive and skip spawning individuals.

Peak spawning of Yakima River steelhead typically occurs in April, with lower elevation subpopulations spawning earlier and higher elevation subpopulations later (Hockersmith et al. 1995, Frederiksen et al. 2015). Thus, plasma E2 and VG levels from October plasma samples from rematuring kelts would reflect ovarian development approximately six months before spawning. To our knowledge, no information is available on plasma E2 or VG levels in anadromous steelhead at a comparable stage of reproductive development. However, in maturing non-anadromous rainbow trout, plasma E2 levels peak approximately four months prior to spawning, whereas plasma VG levels peak approximately one month prior to spawning (Bromage et al. 1982, Whitehead et al. 1983, Tyler et al. 1990, Prat et al. 1996, Wilkinson et al. 2010, Nagler et al. 2012). Therefore, plasma E2 would be approaching maximal levels but plasma VG would be expected to continue to increase for approximately five more months in plasma samples from kelts in October. Considering that peak levels had not yet been attained, rematuring kelt plasma E2 (9-66 ng ml⁻¹) and VG (2-9 mg ml⁻¹) levels in the present study appear to be on the high end of the ranges reported for other anadromous salmonids and non-anadromous rainbow trout, although assay and stock differences make comparison difficult (Kagawa et al. 1983, Fitzpatrick et al. 1986, Eales et al. 1991, Crim et al. 1992, Slater et al. 1994, Pankhurst et al. 2011). Based on these findings, investment of energy into ovarian growth and development appears to be high in rematuring reconditioned steelhead kelts.

Rematuration rates varied widely over the three years of the study, from 25% in 2010 to 65% in 2009. Additional years of data are required before an assessment of a typical rematuration rate can be made or an analysis of drivers of rematuration can be undertaken. However, given that fish culture conditions are relatively constant, the level of variation found suggests that pre-capture environmental conditions influence maturation rate. In our previous

study on survival of Yakima River kelts during reconditioning, we found that high spawner abundance decreased the proportion of the run collected as kelts, and that the proportion of the run collected as kelts was positively correlated with survival during reconditioning, suggesting that spawning competition may negatively impact subsequent survival during reconditioning (Hatch et al. 2013). Similarly, rematuration rates may be influenced by pre-capture migration and spawning conditions such as spawner density and river flows and temperatures, or possibly by feeding and growth conditions in the ocean prior to return.

The present results are consistent with consecutive rematuration rates in captive reconditioning of other anadromous iteroparous salmonids. Female consecutive rematuration rates vary widely in captive reconditioning of Atlantic salmon, ranging from 0-85%, with typical rates in the 20-60% range (Gray et al. 1987, Johnston et al. 1987, Pepper and Parsons 1987, Johnston et al. 1990, Dumas et al. 1991, Crim et al. 1992, Johnston et al. 1992). Approximately 33% of reconditioned wild sea trout (*Salmo trutta*) kelts rematured after one summer (Poole et al. 1994). Natural repeat spawning mid-Columbia steelhead tagged at McNary Dam near the Yakima River confluence were 47% consecutive and 53% skip spawners (Keefer et al. 2008). Natural repeat spawners presumably divide into rematuring and non-rematuring individuals by the end of their first post-spawning summer in the ocean; however, the cohort of non-rematuring fish remains in the ocean and is subject to an additional year of ocean mortality before returning (e.g. Hubley and Gibson 2011). Therefore, the natural rematuration rate for repeat spawners is probably much less than 50%. Based on these considerations, we hypothesize that both pre-capture environmental conditions and culture conditions may influence rematuration rate.

The migration patterns of kelts after release were consistent with consecutive spawning in rematuring females, and with the potential for skip spawning in non-rematuring females.

Detections of rematuring fish were almost all at PD, approximately 500 meters upstream of the release point. The portion of rematuring fish not detected at PD may have been due to missed detections or post-release mortalities; however, these fish were rarely detected elsewhere. The single out of basin detection in the Walla Walla River could have been from a Walla Walla origin fish that strayed into the Yakima as a maiden spawner and was then captured and reconditioned, or a Yakima origin fish that strayed as a kelt after reconditioning. Detections of non-rematuring fish at PD during the fall and winter after release are likely due to movements of fish seeking winter holding areas. Detections of non-rematuring fish the following summer and fall, albeit at a low rate, indicates that these fish are capable of skip spawning. These detections were presumably from fish that migrated to the ocean and continued reconditioning after release, consistent with non-rematuring fish detections at downstream dam bypasses during the fall and spring after release. The return rate of non-rematuring fish was similar to the natural repeat spawning rate for Yakima steelhead (Hockersmith et al. 1995, Hatch et al. 2013, Trammell et al. 2016), suggesting high mortality during downstream migration and in the estuary and ocean (Branstetter et al. 2011, Null et al. 2013).

Rematuration was strongly and consistently associated with positive energy balance over the course of reconditioning. Rematuring fish grew faster in both length and weight over the reconditioning period compared to non-rematuring fish, and had attained higher muscle lipid levels and K by the release sampling in October. Among both rematuring and non-rematuring fish, muscle lipid levels measured with the microwave energy meter only explained approximately 25% of the variation in K, a commonly used non-lethal index of whole body lipid levels (Sutton et al. 2000), suggesting that substantial inter-individual variation exists in the relationship between muscle and whole body lipid levels in steelhead kelts (Hanson et al. 2010).

395 The higher muscle lipid levels found in rematuring fish suggest that that mobilization of muscle
396 lipids to support ovarian development had not yet erased a greater rate of muscle lipid storage in
397 rematuring versus non-rematuring fish. Increased growth and energy reserves in rematuring kelts
398 may be a cause or a consequence of the maturation process. Elevated growth rate and energy
399 stores are thought to stimulate maturation during the spring initiation decision window in *O.*
400 *mykiss* (Bromage et al. 1992, Satterthwaite et al. 2009, Sloat et al. 2014, Kendall et al. 2015).
401 Growth rate, K, and plasma levels of insulin-like growth factor-1, a growth stimulatory hormone,
402 were increased in maturing versus immature rainbow trout over the year prior to spawning
403 (Taylor et al. 2008). Similarly, body weight gain and Fulton's K were greater in rematuring
404 versus non-rematuring female Atlantic salmon kelts over the reconditioning period (Johnston et
405 al. 1987, Johnston et al. 1990). Increased growth may be due to stimulation of appetite during
406 the early stages of maturation (Kadri et al. 1996, Stead et al. 1999). Atlantic salmon kelt
407 consecutive spawners were reported to initiate feeding before non-spawners (Johnston et al.
408 1987). Fulton's K in rematuring reconditioned kelts was greater than that in maiden female
409 Yakima River steelhead spawners sampled for a radio tagging study in the early 1990s ($0.94 \pm$
410 0.008 , from data in Hockersmith et al. 1995), suggesting greater energy reserves in the
411 reconditioned kelts. The increase in length (1.7 cm) in rematuring female kelts was less than
412 reported for natural consecutive repeat spawning female steelhead from Snow Creek, a coastal
413 winter run stream (4.1 cm, Seamons and Quinn 2010), however, the duration of reconditioning
414 for the Prosser fish (average 173 days) was approximately one half of the 11 months at sea for
415 Snow Creek kelts (Quinn and Myers 2004), and the migration timing and distance for Prosser
416 fish would be expected to result in greater energy depletion. Artificially spawned steelhead kelts
417 released from the Coleman National Fish Hatchery on the Sacramento River increased by an

impressive 7.1 cm in length at return when anadromous, versus 1.6 cm for freshwater resident kelts, illustrating the importance of ocean growth conditions (Null et al. 2013).

The physiological decision to remature appears to be made early in captive reconditioned kelts, likely during the first two months of reconditioning. By mid-June to mid-July, 48 to 78 days after collection, plasma E2 had significantly increased in rematuring but not in non-rematuring kelts, resulting in divergence of the two groups. During the same time period, plasma VG decreased in non-rematuring fish, but not in rematuring fish (in 2 of the 3 years). These findings indicate that the rematuration process is underway in rematuring but not non-rematuring kelts within this time period, refuting our initial hypothesis that all fish might initiate rematuration, and the functional decision point might be later. Consistent with the present results, in post-spawning female rainbow trout, plasma E2 increased in fully fed but not in nutritionally restricted fish within 10 weeks after spawning (Caldwell et al. 2014), and in rematuring female Atlantic salmon kelts, an increase in plasma VG was first detected 8 months prior to spawning (Crim et al. 1992). Elevations in plasma E2 and VG only occur after upstream events in the brain-pituitary-ovary-liver endocrine axis (Lubzens et al. 2010, Taranger et al. 2010, Wootton and Smith 2015). The amount of time required for these events is not known with any certainty; however, in maturing coho and Atlantic salmon maiden females, increases in plasma E2 were first detected 10 months before ovulation (Campbell et al. 2006, Andersson et al. 2013). This suggests that rematuration is determined during the time period around spawning in steelhead kelts, or possibly before spawning. Indeed, in 2009, a significant elevation in E2 was found at collection in kelts that went on to remature. The ovaries of steelhead kelts collected after spawning contained oocytes in stages of previtellogenic growth, from the perinucleolar to early and late cortical alveolar stage (Penney and Moffitt 2014a), similar to findings in coho

salmon one year prior to spawning (Campbell et al. 2006), consistent with initiation of rematuration in the kelts at this time. The early divergence of rematuring and non-rematuring female kelts implies that individual differences in physiological condition during the time period around spawning interact with maturation thresholds to determine life history trajectory, similar to findings in repeat spawning winter flounder (Burton 1994). It is perhaps surprising that this occurs when the fish are maximally depleted in energy stores, and before post-spawning feeding conditions can have had much effect, providing an example of how physiological mechanisms constrain life-history variation (Ricklefs and Wikelski 2002).

There was a large increase in E2 over time in rematuring kelts, whereas VG decreased slowly over time in non-rematuring fish. This difference is probably due to the dynamics of production and clearance of the two factors over the reproductive cycle. Estradiol is low at spawning in salmonids, and is rapidly cleared from the blood by conjugation into water-soluble metabolites in the liver and excretion by the kidney (Pankhurst 2008, James 2011). The increase in E2 in rematuring fish reflects active synthesis of E2 in the theca and granulosa cells in the developing ovary. Plasma levels of VG, in contrast, are very high at spawning, and the primary clearance mechanism for VG is uptake by the developing oocytes (Lubzens et al. 2010, Wootton and Smith 2015). After spawning, this clearance mechanism no longer operates. The slow decrease over time in plasma VG levels in non-rematuring fish reflects gradual clearance of the pool of plasma VG present at spawning by secondary clearance mechanisms (Hemmer et al. 2002, Nagler et al. 2012). Although significant differences in plasma E2 and VG level were found at earlier time points, diagnostic separation of rematuring and non-rematuring individual females with no overlap in plasma E2 or VG level was not consistently attained until the 3rd

sampling point, from August 6th to Sept 8th. Therefore, female kelts can be screened for maturation status based on a blood sample taken from approximately mid-August onward.

Management Implications and Further Research

This study shows that female kelt steelhead can be non-lethally screened for maturation status before release, enabling separate management of consecutive and presumed skip spawners. The high proportion of non-rematuring fish present at release in some years and the low return rate of these fish present an opportunity to increase the benefit of the Prosser reconditioning project by putting these females on the spawning grounds the next year. Further research evaluating management strategies to increase the contribution of non-rematuring fish is needed. Leading options include holding fish for an additional year of reconditioning, or transporting and releasing fish to facilitate access to the ocean. Atlantic salmon female kelt rematuration rates increased to 83-100% when fish were held for a second year (Gray et al. 1987, Johnston et al. 1990, Crim et al. 1992), suggesting that this is likely to be an effective strategy, albeit at the cost of the additional effort and mortality involved with holding fish for a second year. Detections of non-rematuring fish migrating downstream during the fall and spring suggest that transport and release has potential. However, for this to be an effective strategy, transport would have to substantially improve the 2.9% return rate found for non-rematuring fish released into the Yakima River in the fall.

Any spawning by viable reconditioned kelts after release will contribute to natural steelhead production. Nevertheless, to justify the effort and expense involved in reconditioning steelhead kelts, project proponents must attempt to quantify this contribution, which requires estimation of the relative reproductive success of reconditioned kelts as compared to maiden spawners. We believe that this difficult task is best approached using a combination of in-river

studies using genetic parentage analysis, studies of kelts in spawning in semi-natural environments such as spawning channels, and studies using a hatchery steelhead kelt model (Hatch et al. 2016). The high plasma E2 and VG levels found in rematuring reconditioned kelts in the present study suggest that reproductive investment is high in these fish. However, to confirm that reproductive development is on track and kelts have sufficient energy reserves to spawn successfully, reproductive development and energy reserves should be directly compared between reconditioned kelts and maidens at a similar stage of their spawning migration. In addition, kelts increased in length during reconditioning, which would be expected to result in an increase in fecundity (Quinn 2005, Seamons and Quinn 2010). Additional growth and a further increase in fecundity would be expected for non-rematuring fish held for a second year that remature as skip spawners. Further hatchery based research is needed to quantify aspects of reproductive performance such as fecundity, egg size, egg quality, and spawn timing in maiden spawners versus consecutive and skip spawning reconditioned kelts. This should be extended to an analysis of relationships between metrics of reproductive development and energy reserves in reconditioned kelts, and subsequent reproductive performance and success in the hatchery, semi-natural, and natural environments. Quantification of the reproductive performance of reconditioned kelts will also illustrate the potential of an alternative use of reconditioning technology: the use of reconditioned fish as broodstock for traditional hatchery production.

Maturation decisions appear to be made early in steelhead kelt reconditioning, and the high variation in maturation rate between years suggests that pre-capture conditions influence maturation rate. Understanding when the maturation decision is made and what the inputs to the decision are would enable project managers to focus collection and husbandry efforts appropriately to maximize project benefits. In particular, the association between growth and

maturation found in the present study requires further analysis. Does increased feeding and growth precede and stimulate rematuration, or does initiation of rematuration precede and stimulate feeding and growth, or both? Depending on how this works, it may be possible to develop interventions to stimulate consecutive rematuration, or screening tools for selecting fish at collection based on predicted life history trajectory. Further research is needed on the physiological mechanisms linking growth and reproductive maturation, and on the relationship between physiological condition at collection and subsequent life history trajectory.

Acknowledgements

The Prosser steelhead kelt reconditioning project is the result of cooperative efforts by many individuals from a variety of agencies, including the Yakama Nation Fisheries, the Columbia River Inter-Tribal Fish Commission, the U.S. Bureau of Reclamation, the U.S. Fish and Wildlife Service, the Pacific States Marine Fisheries Commission, the National Marine Fisheries Service, and the University of Idaho. We would especially like to thank the many managers, biologists, technicians, and staff who have contributed to the protection of Yakima Basin steelhead through the years. Thanks to Denise Kelsey for creating the Yakima Basin map. We are grateful for the perceptive comments of two anonymous reviewers which improved this manuscript. This work was funded by the Bonneville Power Administration (Project 2007-401-00), through the Columbia Basin Fish Accords Agreement. Fish care and sampling were conducted in accordance with animal care guidelines and practices of the Yakama Nation, and under a protocol reviewed and approved by the University of Idaho Animal Care Committee (2010-8).

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Table 1. Numbers of female kelts with known maturation status released and detected post-release migrating toward spawning areas as consecutive and skip spawners.

Release Year	Maturation	Fish Released	Consecutive Spawning Detections ^a	Rate (%)	Fisher's Exact Test <i>P</i> value ^b	Skip Spawning Detections ^c	Rate (%)
2009	Rematuring	49	18	36.7	0.007	0	0.0
	Non-rematuring	26	2	7.7		2	7.7
2010	Rematuring	4 ^d	3	75.0	0.061	0	0.0
	Non-rematuring	61	15	24.6		1	1.6
2011	Rematuring	70	47	67.1	<.001	1 ^e	1.4
	Non-rematuring	53	9	17.0		1	1.9
All	Rematuring	123	68	55.3	<.001	1	0.8
	Non-rematuring	140	26	18.6		4	2.9

Note: Significant ($\alpha=0.05$) *P* values are bolded.

^aConsecutive Spawning Detections were detections from release through June 30th of the following year.

^bTest of whether Consecutive Spawning Detections differed between Rematuring and Non-rematuring fish. Skip Spawning Detections were not included in the contingency test because the number of detections was not sufficient.

^cSkip Spawning Detections were detections from July 1st of the following year onward.

^dMost known rematuring kelts were retained for an experiment instead of being released in 2010.

^eThis fish was first detected as a consecutive spawner in November 2011, and then as a skip spawner in August 2013.

Table 2. Results GLM analysis assessing effects of maturation status, year, and interactions on growth during reconditioning and energy reserves at the release sampling in reconditioned female kelts.

Response	Effect	df	χ^2	<i>P</i>
SGRL ^a (% day ⁻¹)	Maturation	1	60.63	<.001
	Year	2	20.08	<.001
	Maturation x Year	2	4.34	0.114
SGRW ^b (% day ⁻¹)	Maturation	1	72.34	<.001
	Year	2	3.11	0.211
	Maturation x Year	2	8.66	0.013
Muscle Lipid (%)	Maturation	1	46.27	<.001
	Year	2	36.63	<.001
	Maturation x Year	2	2.97	0.227
Fulton's K	Maturation	1	54.94	<.001
	Year	2	1.16	0.559
	Maturation x Year	2	1.79	0.408

Note: Significant ($\alpha=0.05$) *P* values are bolded.
^aSpecific Growth Rate in Length, measured from collection to release.
^bSpecific Growth Rate in Weight, measured from collection to release.

Table 3. Correlation matrix of *R* values (above dashes) and associated unadjusted *P* values (below dashes) for release growth and energy metrics and plasma E2 and VG levels in rematuring and non-rematuring reconditioned female kelts.

		SGRL ^a (% day ⁻¹)	SGRW ^b (% day ⁻¹)	Muscle Lipid (%)	Fulton's K	log E2 (pg ml ⁻¹)	Log Vg (mg ml ⁻¹)
Rematuring	SGRL	-	0.580	0.558	0.433	0.157	-0.054
	SGRW	<.001	-	0.565	0.847	0.250	0.012
	Muscle Lipid	<.001	<.001	-	0.527	0.070	-0.124
	Fulton's K	<.001	<.001	<.001	-	0.251	0.037
	Log E2	0.043	0.001	0.367	0.001	-	0.614
	Log VG	0.483	0.877	0.107	0.635	<.001	-
Non-Rematuring	SGRL	-	0.595	0.643	0.371	0.429	0.202
	SGRW	<.001	-	0.587	0.871	0.583	0.218
	Muscle Lipid	<.001	<.001	-	0.491	0.414	0.211
	Fulton's K	<.001	<.001	<.001	-	0.449	0.257
	Log E2	<.001	<.001	<.001	<.001	-	0.326
	Log VG	0.006	0.003	0.004	<.001	<.001	-

Note: Significant ($\alpha=0.05$) *R* and associated *P* values are bolded. Years were combined prior to analysis.

^aSpecific Growth Rate in Length, measured from collection to release.

^bSpecific Growth Rate in Weight, measured from collection to release.

Table 4. Results of GLM analysis assessing effects of maturation status, sample date, and interactions on plasma estradiol (E2) and vitellogenin (VG) levels in serially sampled female kelts during reconditioning.

Year	Response	Effect	df	χ^2	<i>P</i>
2009	E2	Maturation	1	116.71	<.001
		Sample Date	3	93.78	<.001
		Maturation x Date	3	41.68	<.001
	VG	Maturation	1	95.38	<.001
		Sample Date	3	11.86	0.008
		Maturation x Date	3	66.38	<.001
2010	E2	Maturation	1	56.88	<.001
		Sample Date	3	67.09	<.001
		Maturation x Date	3	51.24	<.001
	VG	Maturation	1	48.91	<.001
		Sample Date	3	32.31	<.001
		Maturation x Date	3	31.93	<.001
2011	E2	Maturation	1	202.79	<.001
		Sample Date	3	138.62	<.001
		Maturation x Date	3	134.52	<.001
	VG	Maturation	1	202.38	<.001
		Sample Date	3	25.32	<.001
		Maturation x Date	3	123.49	<.001

Note: Maturation status was assigned based on cluster analysis of release E2 and VG levels. Collection samples were treated as single Sample Date point. Plasma E2 and VG levels were log transformed prior to analysis. Significant ($\alpha=0.05$) *P* values are bolded.

Figure legends

Fig. 1. Map showing the Yakima River Basin and major steelhead spawning tributaries. The inset shows the location of the Yakima River Basin within the Columbia River Basin on the North American west coast. The Chandler Juvenile Monitoring Facility and Prosser Hatchery are located approximately 500 meters downstream from Prosser Diversion Dam.

Fig. 2. Relationship of plasma levels of estradiol (E2) and vitellogenin (VG) in reconditioned female kelts at pre-release sampling in the fall of 2009 (A: 10/29/2009), 2010 (B: 10/13/2010), and 2011 (C: 10/13/2011). Fish were grouped by cluster analysis (Ward linkage, 2 groups specified, data standardized), and the resulting groups are indicated by circles (rematuring fish with high E2 and VG) and triangles (non-rematuring fish with low E2 and VG). Proposed threshold values for screening fish for maturation status by plasma E2 (500 pg mL^{-1}) and VG (0.1 mg mL^{-1}) level are indicated by lines.

Fig. 3. Necropsies showing ovaries of non-rematuring (A, C) and rematuring (B, D) mortalities after the 10/13/2010 sampling. White arrows indicate ovaries. Plasma E2 (pg mL^{-1}) and VG (mg mL^{-1}) levels were A: 81.4 and $2.4\text{e-}3$; B: 9073 and 5.3; C: 59.2 and $7.4\text{e-}6$; D: 14922 and 21.8; respectively.

Fig. 4. Specific growth rates in length (A) and weight (B), release sampling muscle lipid levels (C) and Fulton's Condition Factor (D) in rematuring and non-rematuring female steelhead kelts. Whiskers show data range, bars the interquartile range, and lines the median. Asterisks indicate significant differences between rematuring and non-rematuring fish within years, and are shown for all years to illustrate the consistent pattern (Tukey's HSD test, $p < 0.05$). Upper case letters above the year label indicate significant differences among years with rematuring and non-rematuring fish combined, and lower case letters to the right of bars indicate significant

differences among years within rematuring and non-rematuring fish. Years or bars not sharing a letter differ significantly (Tukey's HSD test, $p < 0.05$).

Fig. 5. Plasma levels of estradiol (E2) and vitellogenin (VG) in female kelts from collection for reconditioning to release in 2009 (A,B), 2010 (C,D), and 2011 (E,F). Open bars show

rematuring kelts and striped bars show non-rematuring kelts. Whiskers show data range, bars the interquartile range, and lines the median. Asterisks indicate significant differences between

rematuring and non-rematuring kelts (Tukey's HSD test, $p < 0.05$), and letters significant differences over time within the rematuring and non-rematuring groups (Tukey's HSD test, a-c

non-rematuring kelts, x-z rematuring kelts, bars not sharing a letter differ significantly at $p < 0.05$). Collection data were pooled for all surviving serially sampled fish within each year

(Collection date ranges 4/9/2009 to 6/3/2009, 6/3/2010 to 6/11/2010, 4/13/2011 to 5/16/2011).

139x106mm (300 x 300 DPI)

221x203mm (300 x 300 DPI)

215x279mm (150 x 150 DPI)

192x143mm (300 x 300 DPI)

194x209mm (300 x 300 DPI)