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

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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-17β (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.

Introduction

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

Post-spawning anadromous rainbow trout (steelhead kelts, *Oncorhynchus mykiss*) provide a model for the study of consecutive and skipped spawning. The extensive migration and prolonged freshwater holding of stream-maturing (summer run) steelhead in large river systems such as the Columbia River leads to an extreme energy deficit and high post-spawning mortality (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

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

In the Yakima River in the mid-Columbia Basin, captive steelhead kelt reconditioning is being implemented as a recovery tool for populations listed under the Endangered Species Act (ESA, Branstetter et al. 2011, Hatch et al. 2013, Trammell et al. 2016). The goal of the project is to increase natural steelhead production by increasing the number of repeat spawners. Downstream migrating kelts are collected during the spring at an irrigation diversion at Prosser, Washington, on the lower Yakima River. Fish are treated for diseases and parasites, put into tanks, and fed. Surviving kelts are released into the river in mid-October, with the intention that these fish will migrate upriver to spawn. Kelt collections and releases are dominated by female fish (93%, Hatch et al. 2013). However, the reproductive status of female Prosser kelts at release in October has not been investigated. We hypothesize that consecutive and skip spawning individuals are present, and that rematuration as a consecutive spawner is associated with 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 $NaHCO_3$ 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

168 before use in the RIA protocol, and values were corrected for extraction efficiency (Caldwell et
169 al. 2014). These assays and the extraction procedure have been validated for *O. mykiss* and
170 closely related species (Campbell et al. 2006, Nagler et al. 2012, Caldwell et al. 2014). Intra-
171 assay %CV was 3.4% and 6.6%, and inter-assay %CV was 6.3% and 9.0%, in the high and low
172 range RIAs, respectively. Instead of assigning a zero value, plasma samples below the detection
173 limit of the low range E2 assay (ED 80) or the VG assay (lowest standard used in the standard
174 curve) were assigned a value of one half of the lowest detectable level (E2 2009: 0 samples,
175 2010: 5, 2011: 10; VG 2009: 0, 2010: 9, 2011: 6).

176 *Post-Release Detections*

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

187 *Data Analysis*

188 Analysis was restricted to female fish positively identified by PIT tags that survived
189 reconditioning. Plasma E2 and VG data were $\log_{10}$ transformed prior to analysis. Fish were
190 categorized into two groups at release based on cluster analysis of release plasma E2 and VG

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

214 years. Significant results were further analyzed by 1-way ANOVA and Tukey's HSD test. Data
215 are reported in the text as mean ± standard error. Statistical analyses were conducted using JMP
216 12.0.1 (SAS Institute, Cary, NC).

217 **Results**

218 Cluster analysis of VG and E2 values revealed division of female kelts into two groups at
219 the time of release in October in 2009-2011 (Fig. 2). Threshold values of 0.1 mg ml^{-1} VG and
220 500 pg ml^{-1} E2 separated the two groups at the time of release. Fish in the group with high E2
221 and VG levels were classified as rematuring, and fish in the group with low E2 and VG levels
222 were classified as non-rematuring. Due to insufficient plasma for both assays, a few additional
223 fish were classified based on threshold values of either E2 or VG alone (2009: 5 fish, 2010: 8
224 fish, 2011: 0 fish). The percentage of rematuring female fish at release was 65% in 2009, 25% in
225 2010, and 54% in 2011. Rematuring female VG levels at release were 6.5 ± .5, 9.3 ± 1.1, and 1.7
226 ± .2 mg ml^{-1} in 2009-2011, respectively, whereas E2 levels at release were 66100 ± 5600, 12600
227 ± 1600, and 8700 ± 700 pg ml^{-1}.

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

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

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

418 impressive 7.1 cm in length at return when anadromous, versus 1.6 cm for freshwater resident
419 kelts, illustrating the importance of ocean growth conditions (Null et al. 2013).
420 The physiological decision to remature appears to be made early in captive reconditioned
421 kelts, likely during the first two months of reconditioning. By mid-June to mid-July, 48 to 78
422 days after collection, plasma E2 had significantly increased in rematuring but not in non-
423 rematuring kelts, resulting in divergence of the two groups. During the same time period, plasma
424 VG decreased in non-rematuring fish, but not in rematuring fish (in 2 of the 3 years). These
425 findings indicate that the rematuration process is underway in rematuring but not non-rematuring
426 kelts within this time period, refuting our initial hypothesis that all fish might initiate
427 rematuration, and the functional decision point might be later. Consistent with the present
428 results, in post-spawning female rainbow trout, plasma E2 increased in fully fed but not in
429 nutritionally restricted fish within 10 weeks after spawning (Caldwell et al. 2014), and in
430 rematuring female Atlantic salmon kelts, an increase in plasma VG was first detected 8 months
431 prior to spawning (Crim et al. 1992). Elevations in plasma E2 and VG only occur after upstream
432 events in the brain-pituitary-ovary-liver endocrine axis (Lubzens et al. 2010, Taranger et al. 2010,
433 Wootton and Smith 2015). The amount of time required for these events is not known with any
434 certainty; however, in maturing coho and Atlantic salmon maiden females, increases in plasma
435 E2 were first detected 10 months before ovulation (Campbell et al. 2006, Andersson et al. 2013).
436 This suggests that rematuration is determined during the time period around spawning in
437 steelhead kelts, or possibly before spawning. Indeed, in 2009, a significant elevation in E2 was
438 found at collection in kelts that went on to remature. The ovaries of steelhead kelts collected
439 after spawning contained oocytes in stages of previtellogenic growth, from the perinucleolar to
440 early and late cortical alveolar stage (Penney and Moffitt 2014a), similar to findings in coho

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

449 There was a large increase in E2 over time in rematuring kelts, whereas VG decreased
450 slowly over time in non-rematuring fish. This difference is probably due to the dynamics of
451 production and clearance of the two factors over the reproductive cycle. Estradiol is low at
452 spawning in salmonids, and is rapidly cleared from the blood by conjugation into water-soluble
453 metabolites in the liver and excretion by the kidney (Pankhurst 2008, James 2011). The increase
454 in E2 in rematuring fish reflects active synthesis of E2 in the theca and granulosa cells in the
455 developing ovary. Plasma levels of VG, in contrast, are very high at spawning, and the primary
456 clearance mechanism for VG is uptake by the developing oocytes (Lubzens et al. 2010, Wootton
457 and Smith 2015). After spawning, this clearance mechanism no longer operates. The slow
458 decrease over time in plasma VG levels in non-rematuring fish reflects gradual clearance of the
459 pool of plasma VG present at spawning by secondary clearance mechanisms (Hemmer et al.
460 2002, Nagler et al. 2012). Although significant differences in plasma E2 and VG level were
461 found at earlier time points, diagnostic separation of rematuring and non-rematuring individual
462 females with no overlap in plasma E2 or VG level was not consistently attained until the 3rd

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

465 **Management Implications and Further Research**

466 This study shows that female kelt steelhead can be non-lethally screened for maturation
467 status before release, enabling separate management of consecutive and presumed skip spawners.
468 The high proportion of non-rematuring fish present at release in some years and the low return
469 rate of these fish present an opportunity to increase the benefit of the Prosser reconditioning
470 project by putting these females on the spawning grounds the next year. Further research
471 evaluating management strategies to increase the contribution of non-rematuring fish is needed.
472 Leading options include holding fish for an additional year of reconditioning, or transporting and
473 releasing fish to facilitate access to the ocean. Atlantic salmon female kelt rematuration rates
474 increased to 83-100% when fish were held for a second year (Gray et al. 1987, Johnston et al.
475 1990, Crim et al. 1992), suggesting that this is likely to be an effective strategy, albeit at the cost
476 of the additional effort and mortality involved with holding fish for a second year. Detections of
477 non-rematuring fish migrating downstream during the fall and spring suggest that transport and
478 release has potential. However, for this to be an effective strategy, transport would have to
479 substantially improve the 2.9% return rate found for non-rematuring fish released into the
480 Yakima River in the fall.

481 Any spawning by viable reconditioned kelts after release will contribute to natural
482 steelhead production. Nevertheless, to justify the effort and expense involved in reconditioning
483 steelhead kelts, project proponents must attempt to quantify this contribution, which requires
484 estimation of the relative reproductive success of reconditioned kelts as compared to maiden
485 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.

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727
728

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

Release Year	Maturation	Fish Released	Consecutive Spawning Detections[a]	Rate (%)	Fisher's Exact Test *P* 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

731 **Note:** Significant (α=0.05) *P* values are bolded.
732 [a]Consecutive Spawning Detections were detections from release through June 30th of the
733 following year.
734 [b]Test of whether Consecutive Spawning Detections differed between Rematuring and Non-
735 rematuring fish. Skip Spawning Detections were not included in the contingency test because the
736 number of detections was not sufficient.
737 [c]Skip Spawning Detections were detections from July 1st of the following year onward.
738 [d]Most known rematuring kelts were retained for an experiment instead of being released in 2010.
739 [e]This fish was first detected as a consecutive spawner in November 2011, and then as a skip
740 spawner in August 2013.
741

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

Response	Effect	df	X^2	*P*
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

745 **Note:** Significant (α=0.05) *P* values are bolded.
746 [a]Specific Growth Rate in Length, measured from collection to release.
747 [b]Specific Growth Rate in Weight, measured from collection to release.
748
749
750

751 **Table 3.** Correlation matrix of *R* values (above dashes) and associated unadjusted *P* values
752 (below dashes) for release growth and energy metrics and plasma E2 and VG levels in
753 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**	-

754 **Note:** Significant (α=0.05) *R* and associated *P* values are bolded. Years were combined prior to
755 analysis.
756 [a]Specific Growth Rate in Length, measured from collection to release.
757 [b]Specific Growth Rate in Weight, measured from collection to release.
758

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

Year	Response	Effect	df	X^2	*P*
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**

762 **Note:** Maturation status was assigned based on cluster analysis of release E2 and VG levels.
763 Collection samples were treated as single Sample Date point. Plasma E2 and VG levels were log
764 transformed prior to analysis. Significant (α=0.05) *P* values are bolded.
765
766

767 **Figure legends**

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

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

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

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

790 differences among years within rematuring and non-rematuring fish. Years or bars not sharing a
791 letter differ significantly (Tukey's HSD test, $p < 0.05$).
792 Fig. 5. Plasma levels of estradiol (E2) and vitellogenin (VG) in female kelts from collection for
793 reconditioning to release in 2009 (A,B), 2010 (C,D), and 2011 (E,F). Open bars show
794 rematuring kelts and striped bars show non-rematuring kelts. Whiskers show data range, bars the
795 interquartile range, and lines the median. Asterisks indicate significant differences between
796 rematuring and non-rematuring kelts (Tukey's HSD test, $p < 0.05$), and letters significant
797 differences over time within the rematuring and non-rematuring groups (Tukey's HSD test, a-c
798 non-rematuring kelts, x-z rematuring kelts, bars not sharing a letter differ significantly at $p <$
799 0.05). Collection data were pooled for all surviving serially sampled fish within each year
800 (Collection date ranges 4/9/2009 to 6/3/2009, 6/3/2010 to 6/11/2010, 4/13/2011 to 5/16/2011).
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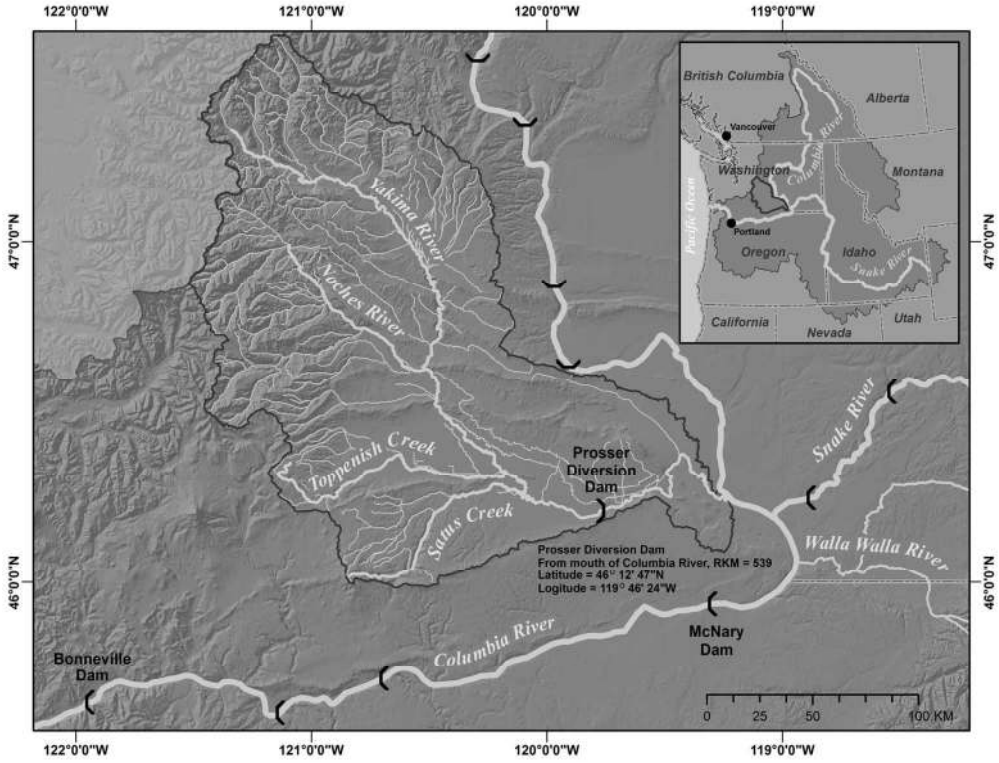

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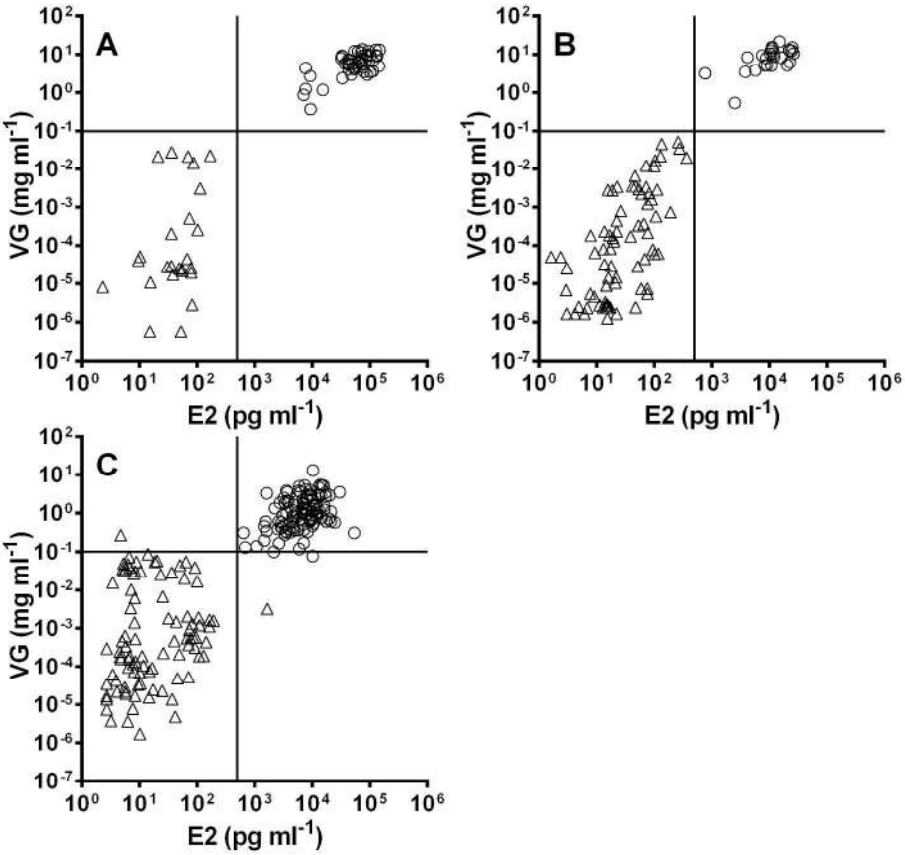

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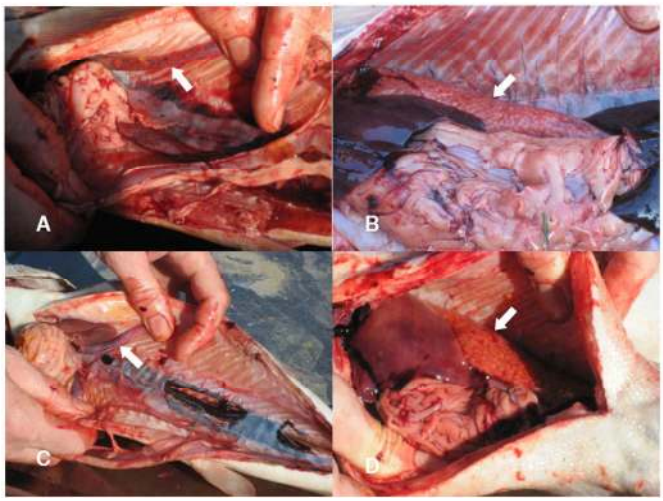

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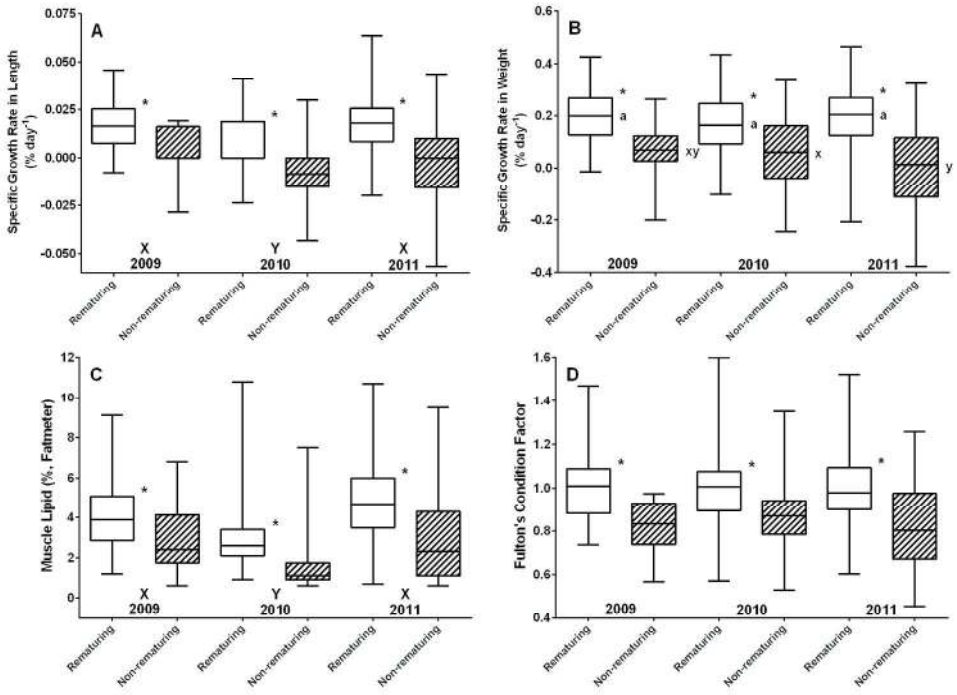

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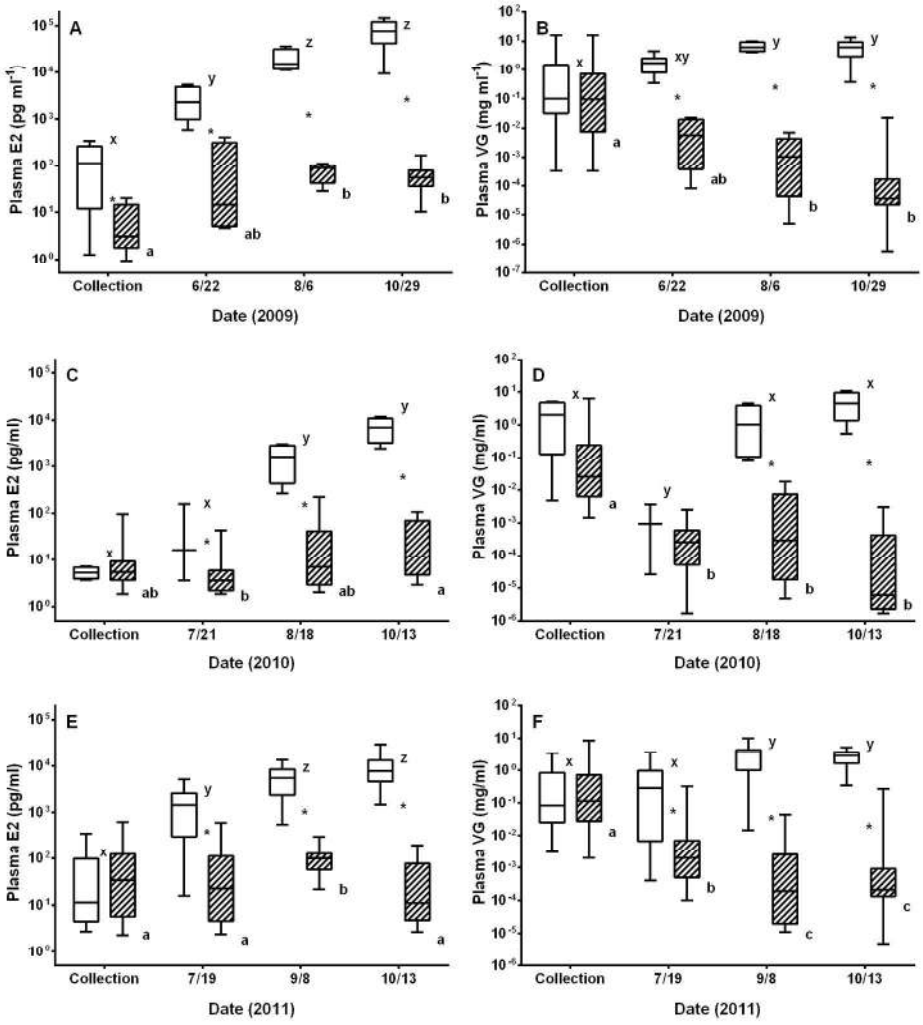

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