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Evaluation of endocrine and transcriptomic markers of male maturation in winter-run Steelhead Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Steelhead Trout (*Oncorhynchus mykiss*) display a varied life-history, including precocious male maturation at age-1 or age-2. In wild fish, precocious male maturation represents an important component of a diverse life-history portfolio. In hatchery programs, however, it is undesirable if rearing practices increase rates of early male maturation and reduce numbers of anadromous male adults. Our study aimed to develop endocrine and molecular markers for identifying males at early stages of maturation in the spring (prior to smolt release) and evaluated the potential use of these markers for quantifying early male maturation rates at a hatchery scale. In a laboratory study, Skookumchuck winter-run Steelhead Trout were reared at a high growth rate in order to increase the occurrence of precocious male maturation. Fish were lethally sub-sampled in February, prior to the time of smolt release; in May, at the time of smolt release; and in September, when 1+ age maturing males that would spawn the following spring were clearly identifiable based solely on gonadosomatic index (GSI). In February and May samples, we measured GSI, plasma 11-ketotestosterone (11KT), mRNAs for pituitary follicle stimulating hormone (*fshb*) and luteinizing hormone (*lhb*) beta subunits, and analyzed stage of spermatogenesis by testis histology. Additionally, in May, we measured testis anti-Müllerian hormone (*amh*) and insulin-like growth factor 3 (*igf3*) mRNA. Our primary goal was to evaluate the aforementioned maturation indices for their efficacy in forecasting the proportion of fish initiating early male maturation in the spring (approximately 1 year prior to spermiation), compared to the proportion that actually matured. Combining measures of GSI, plasma 11KT, and pituitary *fshb* and *lhb* mRNA expression provided a useful, but conservative, estimate of the proportion of males initiating maturation in the spring (21%) compared to the proportion that were ultimately destined to mature (37%) the following spring. These results suggest that maturation may be less synchronous than previously appreciated and some males may have initiated maturation after our census in May.

1. Introduction

In the Pacific Northwest of the United States and Canada, native salmonid species have declined significantly in recent decades due to habitat degradation, fisheries, and hydropower development, among other factors (Lichatowich, 1999). To mitigate for these losses, hatchery programs have been implemented to aid in recovery of stocks listed as endangered under the federal Endangered Species Act (ESA) and to provide fish for harvest and recreational fisheries (HSRG, 2009). In salmonids, the age at which males mature is plastic and influenced by genetic and environmental factors (e.g. photoperiod, temperature, ration) and their interaction (Hazel et al., 1990; Hutchings, 2011; Sloat et al., 2014). Like many other salmonid species, steelhead (*Oncorhynchus mykiss*) exhibit a life-history that includes precocious male maturation (Kendall et al., 2015). Wild steelhead typically spend from 1 to 3 years in freshwater before undergoing the physiological process of smoltification (Hoar, 1988) and outmigrating to the ocean, where they spend 2–3 years in seawater before returning to their natal stream to spawn (Peven et al., 1994). Rather than migrate to the ocean, precocious males remain in freshwater and employ a 'sneaker' strategy to mate with returning full size anadromous females (Kendall et al., 2015). While precocious male maturation represents a viable reproductive strategy within the context of a complex life-history portfolio for wild salmonids (Moore et al., 2014; Schindler et al., 2010; Waples et al.,

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2008), unnaturally high rates in hatchery programs are generally undesirable.

Rearing conditions that accelerate growth or alter body fat levels have been shown to increase rates of precocious male maturation in Chinook Salmon (*O. tshawytscha*) (Larsen et al., 2006; Shearer et al., 2006; Shearer and Swanson, 2000; Silverstein et al., 1998), Atlantic Salmon (*Salmo salar*) (Thorpe, 1994) and Steelhead Trout (Sharpe et al., 2007). Since Steelhead Trout hatchery programs typically rear fish at relatively high growth rates to promote smoltification at one year of age, they also tend to promote high proportions of precocious male maturation at age-1 or -2 years (Berejikian et al., 2012; Sharpe et al., 2007; Tatara et al., 2019). Precocious males may residualize in natal streams where they can potentially compete for resources and interbreed with wild, native fish. High proportions of precociously maturing males can ultimately reduce the number of more desirable large anadromous adults (Hausch and Melnychuk, 2012; Snow et al., 2013). In order to assess and develop rearing practices to control early male maturation at a hatchery scale, it is necessary to develop methods to quantify the proportion of males initiating puberty at the time of smolt releases in the spring, one year prior to reaching full maturity.

Puberty is the developmental period during which the brain-pituitary-gonad axis is functionally competent and a vertebrate animal first becomes capable of reproducing (reviewed by Taranger et al., 2010). For semelparous Pacific salmon, puberty is the process of completing the first and only reproductive period, while in iteroparous species, such as Rainbow Trout and Steelhead Trout, puberty is the completion of the first reproductive cycle. In males, the onset of puberty involves the development of secondary sex characters and appropriate mating behavior, in addition to the initiation of spermatogenesis and eventual production of mature spermatozoa. This process is regulated by reproductive hormones, including pituitary gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) and the primary male androgen, 11-ketotestosterone (11KT; Schulz et al., 2010; Taranger et al., 2010). In Chinook Salmon males that have initiated puberty, pituitary FSH and plasma 11KT levels are elevated (Campbell et al. 2003; Shearer and Swanson, 2000). Similar results have been obtained for Atlantic Salmon (Maugars and Schmitz, 2008a,b; Melo et al., 2015) and Rainbow Trout (Gomez et al., 1999; Kusakabe et al., 2006). FSH regulates expression of a variety of genes in the testis during early stages of spermatogenesis in teleost fish (Nobrega et al., 2015; Sambroni et al., 2013a,b) including, but not limited to, anti-Müllerian hormone (AMH) (Miura et al., 2002; Skaar et al., 2011) and insulin-like growth factor-3 (IGF3) (Melo et al., 2015; Nobrega et al., 2015; Sambroni et al., 2013a,b). AMH inhibits spermatogonial proliferation and IGF3 up-regulates the expression of genes related to spermatogonial differentiation (Nobrega et al., 2015; Skaar et al., 2011). Taken together, alterations in plasma 11KT, pituitary gene expression for the beta subunit of FSH (*fshb*) and LH (*lhb*), and testis gene expression for *amh* and *igf3* provide a suite of candidate markers to identify males initiating puberty in teleost fish.

Previous studies of Chinook Salmon established that plasma 11KT levels in yearling males can be used to estimate the proportion of age-2 maturing males (colloquially referred to as minijacks) in a population when fish are sampled within six weeks of release from the hatchery as smolts (March-April) (Larsen et al., 2004; Medeiros et al., 2018). This time frame is 5–7 months prior to expected spawning of mature fish in September-October of the same year. To accomplish the same objective for winter-run Steelhead Trout, earlier indices of maturation are needed because smolt releases occur approximately 10–12 months in advance of when these fish could spawn if maturing at age-2 (March-April of the following year). In a previous investigation, many of the aforementioned physiological indices of male maturation were used to demonstrate life-history diversity in yearling male Steelhead Trout at a single point in time prior to release from the Parkdale Hatchery, Parkdale, OR, USA (Larsen et al., 2017). This previous study provided useful preliminary results on the suitability of these various markers to characterize diversity of male reproductive phenotypes in a hatchery. Unfortunately, the relationship between forecasted and actual rates of age-2 male maturation could not be determined because the fish were released into the wild and were no longer accessible for subsequent validation of forecasted maturation rates. Therefore, in the current investigation, we conducted a laboratory-based study in captively-reared fish to characterize reproductive status in yearling male Steelhead Trout prior to smolting, at the time of smolt release, and the subsequent fall (6–7 months prior to spawning in this stock of fish).

Laboratory-raised Steelhead Trout were reared under a relatively high growth rate to increase the prevalence of precocious male maturation at age-2. Approximately 15% of the total fish reared were lethally sampled at three time points to monitor frequency distributions of gonadosomatic index (GSI), plasma 11KT, pituitary *fshb* and *lhb* expression, testis *amh* and *igf3* expression, and stage of spermatogenesis by histology. Fish were sampled in February as sub-yearlings and in May as yearlings, which would be prior to and at the time of smolt releases from the hatchery, respectively. The remaining 1+ age fish were sampled in September when spermatogenesis in maturing fish was sufficiently advanced as indicated by GSI (Gomez et al., 1999). The objectives of this study were: 1) to determine if male puberty onset in yearling Steelhead Trout could be detected in the spring using endocrine, transcriptomic, and histological indices; and 2) to compare estimates of the proportion of age 1+ precocious males in September (6–7 months prior to spawning) to those based on the aforementioned markers quantified in May when smolts are generally released from hatcheries. We hypothesized that males initiating spermatogenesis would have higher plasma 11KT, pituitary *fshb* and *lhb*, and testis *igf3* expression, and lower *amh* expression relative to pre-pubertal fish, and that combined measures of these markers would allow us to confirm puberty onset.

2. Materials and methods

2.1. Fish rearing conditions

Steelhead Trout embryos, at the 'eyed' stage of development, were sourced from the Skookumchuck River stock in western Washington State, USA, and were produced by artificially spawning hatchery adults returning in the winter of 2009. The embryos were incubated at the Washington Department of Fish and Wildlife's Bingham Creek Hatchery (Grays Harbor County, WA, 47°8′45.8844″N 123°24′2.8944″W), and upon reaching the 'button-up' stage (May 2009) were transferred to the Northwest Fisheries Science Center's (NWFSC) Manchester Research Station (Kitsap County, WA, 47°34′8.5512″N 122°33′18.306″W). Fry were raised in duplicate 1.2 m diameter outdoor circular tanks until October of 2009 when 300 parr (5.9 g mean weight) were distributed into each of 10 (1.2 m diameter) tanks containing approximately 700 L each. All tanks received a constant flow of well water (10–13 °C) at a rate of 15 L/min. Fish were fed to satiation 5 days per week, throughout the experiment, on a commercial salmon diet (Bio-Oregon, Longview, WA) according to manufacturer's recommendations. In 2010, fish were removed from each tank during February, May, and September for lethal sampling to obtain fork length, body and gonad weight, plasma, and tissues for physiological analyses. In February and May, 35 fish were sampled per tank for a total of 350 each month. In September, all remaining fish were sampled, 23–46 per tank for a total of 330 fish. All fish were reared and sampled according to the University of Washington Institutional Animal Care and Use Committee (Protocol #2313–90).

2.2. Fish sampling procedure

During each sampling event, fish were randomly collected from each tank and held under aeration in a large cooler. Individual fish were anesthetized with buffered 0.05% tricaine methanesulfonate (MS-222,

Table 1
Probe and primer design for quantitative real time RT-PCR assays with calculated assay efficiencies.

Target	Primer or Probe	Sequence (5′-3′)	Amplicon size (bp)	Average PCR efficiency (%)
fshb[††]	Forward primer	AGGACTGTCACGGAAGCATCA	65	102.5
	Reverse primer	GTTCAGGTCCGTTGTTTCGC		
	Probe	TCACCACCTGCGCCGGCC		
lhb[†]	Forward primer	GTCACCAAGGAGCCGGTTTT	69	94.2
	Reverse primer	GTCCCGGTAGGTGCACACA		
	Probe	AGCCCATTTTCCACCGTGTACCAGC		
amh[†††]	Forward primer	CATCTACAACTGCCAGGGAGTCT	72	107.9
	Reverse primer	CTGTTAAGCAGGATAGCATGGT		
	Probe	CAGCTTTCCCCTGACCAACGGGAA		
igf3[††]	Forward primer	ACTGCGCAAAGCCAAAGC	74	101.5
	Reverse primer	GAAATTGCTCCTCCATAACTTGCT		
	Probe	AGCCGACGACACGCTCCCCTC		
ef1a[†]	Forward primer	GAGATGGGCAAGGGCTCTTT	74	107.5
	Reverse primer	GTGATACCACGCTCCCTCTCA		
	Probe	TCAGCTTGTCCAGCACCCAGGCA		

All probes had 6FAM as the 5′ fluorescent reporter dye and BHQ1 as the non-fluorescent 3′ quencher.
[†] Quantitative real time RT-PCR assays for *lhb* and *ef1a* were previously reported by Luckenbach et al. (2010).
[††] Primers and probes for *fshb* and *igf3* quantitative real time RT-PCR assays were designed to rainbow trout sequence data (GenBank Accession numbers: *fshb*, NM001124586; *igf3*, XM023990971).
[†††] Primers and probe for *amh* quantitative real time RT-PCR assay were designed to Coho salmon (*Oncorhynchus kisutch*) sequence data and sequence identity was verified for rainbow trout (GenBank Accession number XM021603833).

Argent Chemical Laboratories, Redmond, WA) and euthanized by decapitation. Prior to blood collection, fish were measured for fork length (mm) and weighed (g) to the nearest 0.1 g. After severing the caudal peduncle with a single-edge razor blade, blood was collected from the caudal vasculature using pre-heparinized Natelson tubes (Fisher Scientific, Hampton, NH). Blood samples were stored on ice until sex could be determined macroscopically. Male blood samples were centrifuged at 3000 × g for 6 min while female samples were discarded; no further data was collected for female fish. Plasma was removed by pipette and frozen on dry ice. Pituitary glands were individually dissected and preserved in 0.5 ml RNA*later* (Ambion, Carlsbad, CA) for analysis of gonadotropin subunit mRNAs. Paired testes were removed and weighed to the nearest 0.001 g. Gonadosomatic Index (GSI) was calculated according to the following equation:

$$GSI = [\text{gonad weight(g)}/\text{body weight(g)}] * 100$$

One testis was preserved in 0.75 ml RNA*later* in a 1.5 ml polypropylene microfuge tube for analysis of mRNAs associated with spermatogenesis. The other testis was fixed in 1 ml of Histochoice (AMRESCO, Solon, OH) in a 1.5 ml polypropylene microfuge tube for histological analysis. Plasma and tissue samples were transported to the NWFSC Montlake laboratory in Seattle, WA for analysis. Plasma samples were stored at −80 °C. Tissues preserved in RNA*later* remained at room temperature for seven days before the solution was removed and tissues were stored at −80 °C. Histological samples remained in Histochoice for seven days before transfer to 70% ethanol for long-term storage at room temperature.

2.3. 11-Ketotestosterone (11KT) assay

Plasma was heat treated prior to assay similar to the method of Schulz et al. (1994). Each sample was diluted 1:2 with sterile water (50 µl plasma + 100 µl water) and heated in a water bath set at 80 °C for one hr. Samples were then centrifuged at 18,000 × g for 6 min and supernatants were stored at −20 °C until assay. 11KT (ng/ml) was measured by enzyme-linked immunosorbent assay (ELISA) similar to the method previously described by Cuisset et al. (1994). Acetylcholinesterase tracer and pre-coated (mouse anti-rabbit IgG) 96-well plates were purchased from Cayman Chemical (Ann Arbor, MI). Primary antibody was provided by Dr. David Kime (University of Sheffield, retired).

2.4. Tissue mRNA analyses

Total RNA for pituitary samples collected in February was isolated using Qiagen's RNeasy Plus spin column kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA for pituitary and testis samples collected in May was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Integrity of the RNA was verified by an optical density (OD) absorption ratio OD 260 nm/OD 280 nm > 1.9 and quantified by spectrophotometry at 260 nm using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Total RNA was diluted with nuclease-free water (BioExpress, Radnor, PA) to 10.0 ng/µl and 30 ng per sample was reverse transcribed with Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription (RT) reaction conditions were as follows: 3.0 µl of 5X buffer, 1.5 µl of 0.1 M dTT, 0.75 µl of dNTPs (stock of 10 mM each dCTP, dGTP, dTTP and dATP; Promega, Madison WI), 0.225 µl random hexamers (500 ng/µl stock; Promega), 0.1875 µl Superscript II (200 U/µl; Invitrogen), 0.3 µl RNase inhibitor (20 U/µl; Promega), 6.0375 µl nuclease-free water (BioExpress) and 3.0 µl of template. RT reactions were conducted in an MJ Research PTC-200 (MJ Research, Hercules, CA) with the following temperature profile: 23 °C for 10 min, 48 °C for 60 min and 95 °C for 10 min, followed by a 4 °C incubation. Samples were diluted 1:1 with nuclease-free water (10 µl cDNA + 10 µl water) prior to measurement in real-time quantitative polymerase chain reaction (qPCR) assays.

Primers and probes for real-time RT-qPCR assays were designed according to sequence data using Primer Express 1.5 software [Applied Biosystems (ABI), Foster City, CA] and purchased from Integrated DNA Technologies (San Jose, CA). When possible, intron/exon splice junctions were used in primer design to avoid potential signal from contaminating genomic DNA. Primer and probe sequences for *fshb*, *lhb*, *amh*, *igf3*, and a reference gene, elongation factor one alpha (*ef1a*) are presented in Table 1. For each transcript measured, five randomly selected total RNA samples were analyzed without RT reaction to test for amplification due to genomic DNA contamination (no amplification control, NAC). NAC samples showed no, or negligible, DNA contamination with all samples amplifying > 7 cycles (< 1% DNA contribution) beyond those of their reverse transcribed counterparts. No template controls (NTC), consisting of an RT reaction containing no RNA template, were also performed and showed no template contamination in the reagents used.

All assays were run on an ABI 7900 HT Fast Real-Time PCR System

using 384-well plates and TaqMan Universal PCR Master Mix (ABI). PCR efficiency for each transcript was measured using a serial dilution of a testis or pituitary RNA sample from within the sampling month as a reference. Standard curve dilutions were run in triplicate, samples were run in singles. In previous studies we found that the greatest source of variation was biological rather than technical (Larsen et al., 2017) so we opted to run large sample numbers (approximately 170 males per time point). Samples of the same tissue type within a sampling month were analyzed on a single plate, eliminating plate-to-plate variation. Reaction conditions were as follows for 12 µl PCRs: 6.0 µl of TaqMan Universal PCR MasterMix (ABI), 0.22 µl of forward primer (45 µM stock), 0.22 µl of reverse primer (45 µM stock), 0.24 µl of probe (10 µM stock), 2.32 µl nuclease-free water (BioExpress) and 3.0 µl of diluted RT reaction. Cycling parameters were: 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s followed by 60 °C for 1 min.

Stable expression of *ef1a* was observed within each tissue type (data not shown). Therefore, transcript levels were calculated using the serially diluted total RNA sample standard curve and efficiency corrected by the reference gene (*ef1a*) using the method described by Pierce et al. (2004). To avoid interpretation of the data being driven by a single spurious low sample, relative expression values for all mRNAs within a tissue type and sampling month were calculated as follows: 1) the mean of the highest 10% of Cts was found (hereafter referred to as the standardized mean), 2) Cts higher than the standardized mean + 2 standard deviations were removed from further analysis as spuriously low samples, and 3) the lowest remaining efficiency corrected sample was then set to a value of 1.

2.5. Testis histology

Histological analysis of stage of spermatogenesis was performed on all testis samples collected in May to identify fish that had initiated puberty. Fixed testes were processed through a graded series of ethanol and xylene substitute followed by embedding in paraffin. Sections were cut at 5 µm on a standard rotary microtome and stained with hematoxylin and eosin. Stages of spermatogenesis were semi-quantitatively determined, similar to the method previously described in Larsen et al. (2017). However, in this study only four stages of testis development were observed in May as shown in Fig. 1. Stages of spermatogenesis were defined as follows: stage 0, nearly 100% type A undifferentiated spermatogonia with a small amount of type A differentiated spermatogonia (Fig. 1A); stage 1, approximately 50% type A differentiated spermatogonia and 50% type B spermatogonia (Fig. 1B); stage 2, over 90% type B spermatogonia with a small amount of type A spermatogonia (Fig. 1C); stage 3, presence of all meiotic stages of germ cells, approximately 60% primary and secondary spermatocytes, 30% spermatids, and a small amount of type B spermatogonia (Fig. 1D).

2.6. Statistical analysis

Data were analyzed to accomplish two objectives: 1) to determine the earliest sampling event (February or May) in which initiation of puberty could be detected, and 2) to determine the percent of fish initiating puberty within that month for comparison to the percent found to be maturing in September. Data were tested for normality using the D'Agostino-Pearson omnibus K^2 normality test. Comparisons among months were conducted by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test. Differences between lower and upper modes for the September samples were evaluated by unpaired *t*-test. To control for heteroscedasticity, data for GSI, plasma 11KT, pituitary *fshb* and *lhb*, and testis *amh* and *igf3* were $\log_{10}$ transformed prior to statistical analyses. A finite mixture model was used to analyze frequency distributions of the $\log_{10}$ transformed data for GSI, plasma 11KT, pituitary *fshb* and *lhb* expression, and testis *amh* and *igf3* expression. Regression analyses were conducted to determine the best fit line describing the relationships between $\log_{10}$ transformed data for GSI and plasma 11KT in February, May, and September. Regression analyses were also conducted to determine the best fit line describing the relationships between $\log_{10}$ transformed data for GSI and pituitary *fshb* and *lhb* expression and testis *amh* and *igf3* expression in May only. Fish identified by histological staging as stage 0 were classified as "immature", while fish identified as stage 1–3 were classified as "maturing" and were pooled for statistical analyses. Statistical comparisons between immature and maturing fish were conducted using a non-parametric Mann-Whitney *U* test. All data were graphed and statistically analyzed using Prism 6 software (GraphPad Software, La Jolla, CA) with the exception of the finite mixture model analyses, which were performed with Stata/IC 15 software (StataCorp, College Station, TX).

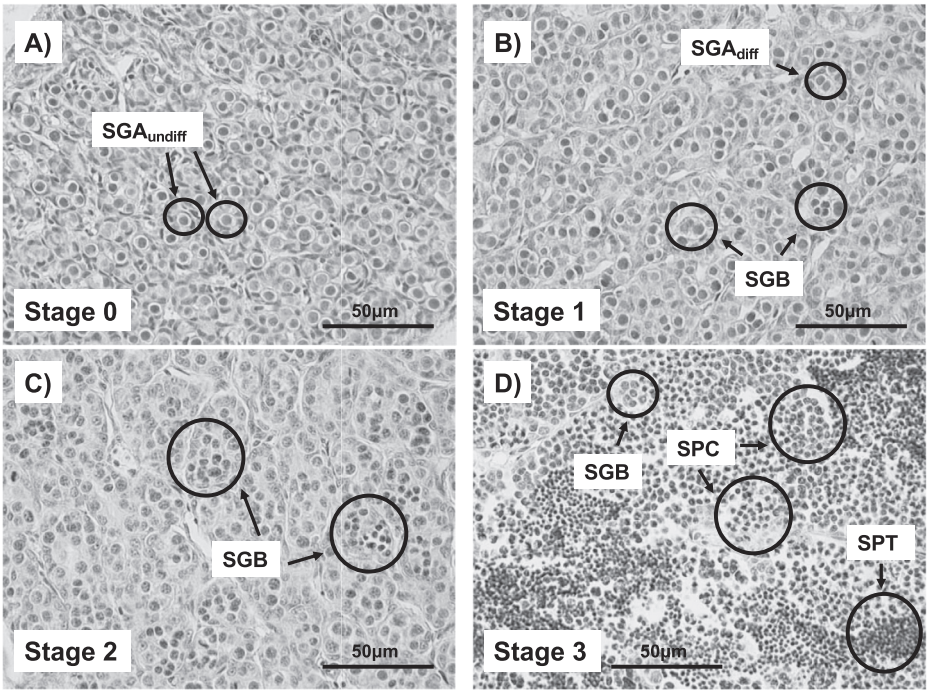

Fig. 1. Sections of testes from Skookumchuck winter-run hatchery steelhead sampled in May 2010. Stages were determined as (A) stage 0, (B) stage 1, (C) stage 2, and (D) stage 3. Abbreviations are as follows: SGA$_{undiff}$, undifferentiated type A spermatogonia; SGA$_{diff}$, differentiated type A spermatogonia; SGB, type B spermatogonia; SPC, spermatocyte; SPT, spermatid. All panels were at 60x magnification.

The level of significance was set at $\alpha = 0.05$.

2.7. Determination of percent of maturing fish

The finite mixture model analyses were largely used to determine the value of delineation between immature and maturing fish in the $\log_{10}$ frequency distributions. Where the BIC value of the finite mixture model with two mixtures (bimodal) was the lowest, we used this model to predict the probability of falling into each of the two modes. We set the boundary between modes to be where the probability = 0.5 (equal chance of falling within each of the two modes). For individual parameters measured, the percent of maturing fish was determined based on the N value of those found to be above (or below in the case of *amh*) the threshold value determined by the finite mixture model analysis, divided by the total N value of males sampled within that sampling event. For combinations of parameters, the percent of maturing fish was determined in the same manner, however, the N value of those found to be above (or below in the case of *amh*) the finite mixture model thresholds was designed to include unique individuals. Therefore, if the same fish was above (or below) a threshold value for more than one parameter within a combination it was not counted more than once.

3. Results

3.1. Body size – February, May, September

Frequency distributions for male fork length and body weight in February, May and September did not show any clear deviation from a unimodal distribution, however the mean and range in body size increased over time (Fig. 2). Fork length ranged from 102 to 189 mm in February, 128 to 269 mm in May, and 178 to 343 mm in September. In February, body weight ranged from 11.6 to 76.5 g. In both May and September, there was nearly a 10-fold range in body weight. In May, body weight ranged from 25.0 to 238.5 g. By September, body weight ranged from 63.3 to 527.4 g. Mean fork length and body weight increased significantly from February through September (Table 2).

3.2. GSI and plasma 11KT – February, May, September

The frequency distributions of male GSI and plasma 11KT levels are shown in Fig. 3. In February, GSI ranged from 0.026 to 0.111% and from 0.013 to 1.897% in May. Although the range was greater in May than February, there was no significant difference in mean GSI between the two months (Table 2). The frequency distribution of GSI in February was clearly unimodal (fmm1 BIC = −259.5, fmm2 BIC = −248.8) (Fig. 3A). In May, the frequency distribution, while not strictly bimodal, was more right-skewed than the February distribution (May skewness = 3.4, February skewness = 0.4). A two-mode model was the best fit, with a split between modes at GSI of 0.054% (fmm2 BIC = −145.7, fmm1 BIC = −14.4) (Fig. 3D). However, by September the frequency distribution of GSI was clearly bimodal (fmm2 BIC = –23.6, fmm1 BIC = 513.6) (Fig. 3G) with means of the lower and upper modes of 0.04% and 7.44%, respectively (Table 2). The distribution in plasma 11KT levels over time showed a similar pattern. In February, plasma 11KT levels ranged from 0.272 to 2.122 ng/ml and in May, plasma 11KT ranged from 0.278 to 3.014 ng/ml. There was no significant difference in mean plasma 11KT levels between February and May (Table 2). The frequency distribution of plasma 11KT in February was clearly unimodal (fmm1 BIC = −215.4, fmm2 BIC = −212.6) (Fig. 3B). However, in May, the distribution, while not strictly bimodal, was more right-skewed than the February distribution (May skewness = 1.2, February skewness = 0.5). A two-mode model was the best fit, with a break between modes at plasma 11KT of 0.81 ng/ml (fmm2 BIC = -153.0, fmm1 BIC = −125.7) (Fig. 3E). By September, there was a distinct bimodal distribution in plasma 11KT levels (fmm2 BIC = 71.7, fmm1 BIC = 221.0) (Fig. 3H) with mean values of the lower and upper modes of 0.64 and 4.59 ng/ml, respectively (Table 2). Linear regressions between GSI and plasma 11KT levels were significant ($p < 0.01$) for all months, but the R^2 values were lower in February ($R^2 = 0.06$) and May ($R^2 = 0.20$), than in September ($R^2 = 0.87$) (Fig. 3C, 3F, 3I). In September, immature and maturing fish, separated based on GSI, clustered tightly and drove the regression relationship between GSI and plasma 11KT (Fig. 3I).

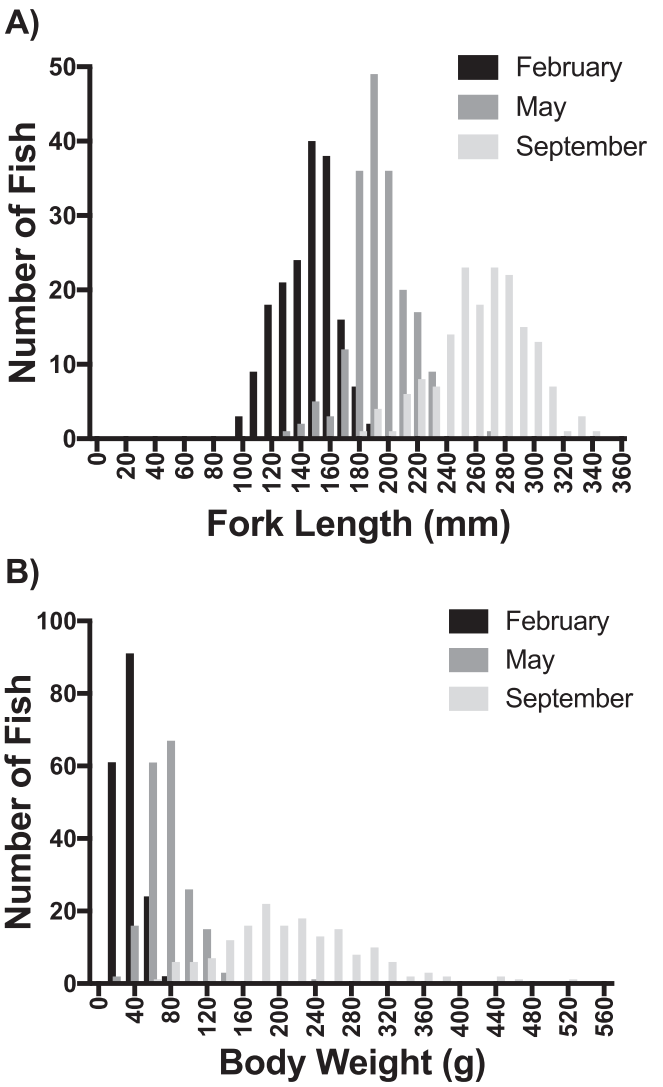

Fig. 2. Frequency distributions of male fork length (A) and body weight (B) for fish sampled in February (N = 178), May (N = 191), and September (N = 167).

3.3. Pituitary gene expression – February and May

Immature and maturing fish were clearly evident in September based on GSI and plasma 11KT levels, therefore the mRNA levels of pituitary *fshb* and *lhb* were measured in males collected during February and May only (Fig. 4) to determine if initiation of maturation was evident in a portion of the males. In February, *fshb* mRNA levels had a 130-fold range and the distribution was clearly unimodal (fmm1 BIC = 196.2, fmm2 BIC = 205.9) (Fig. 4A). Pituitary *lhb* mRNA levels in February had a 400-fold range and, similar to *fshb* levels, did not deviate from a unimodal distribution (fmm1 BIC = 254.5, fmm2 BIC = 258.1) (Fig. 4B). In May, *fshb* mRNA levels had a 260-fold range and the distribution was bimodal with a break between modes at *fshb* relative expression of 64.7 (fmm2 BIC = 280.6, fmm1 BIC = 307.6) (Fig. 4C). Pituitary *lhb* mRNA levels in May had a 400-fold range and

Table 2
Body size, gonadosomatic index (GSI), and plasma 11-ketotestosterone (11KT) levels in male steelhead collected in February, May and September. Data are mean ± standard errors. Superscript letters indicate significant differences ($p < 0.05$) between months. An asterisk indicates a significant difference ($p < 0.05$) between upper and lower modes in September.

Month (N)	Fork Length (mm)	Body Weight (g)	GSI (%)	Plasma 11KT (ng/ml)
February (178)	145.3 ± 1.41[a]	35.97 ± 1.01[a]	0.047 ± 0.001[a]	0.599 ± 0.016[a]
May (191)	193.1 ± 1.41[b]	77.54 ± 1.80[b]	0.050 ± 0.010[a]	0.641 ± 0.025[a]
September (167)	262.6 ± 2.38[c]	214.3 ± 6.06[c]	2.744 ± 0.289[b]	2.084 ± 0.168[b]
September: Lower mode (106)	259.4 ± 2.68	197.9 ± 5.88	0.040 ± 0.001	0.640 ± 0.048
September: Upper mode (61)	268.1 ± 4.51	242.9 ± 12.31[*]	7.442 ± 0.235[*]	4.592 ± 0.206[*]

the data again demonstrated bimodality with a break between modes at *lhb* relative expression of 26.9 (fmm2 BIC = 180.5, fmm1 BIC = 289.0) (Fig. 4D). In May, there were significant positive relationships between $\log_{10}$ GSI and pituitary *fshb* ($R^2 = 0.42$, $p < 0.0001$) (Fig. 5A) and *lhb* mRNA expression ($R^2 = 0.57$, $p < 0.0001$) (Fig. 5B).

3.4. Testis gene expression – May

Since the distribution of pituitary *fshb* and *lhb* gene expression indicated greater variation in reproductive state among the males in May than in February, we examined relative expression of spermatogenesis-related genes in the testis for May samples only. Relative levels of *amh* mRNA expression had a 20-fold range and the frequency distribution demonstrated bimodality with a split between modes at *amh* relative expression of 3.55 (fmm2 BIC = −90.3, fmm1 BIC = −61.2) (Fig. 6A). Testis *igf3* mRNA relative expression showed a 30-fold range and the frequency distribution also demonstrated bimodality with a split between modes at *igf3* relative expression of 5.01 (fmm2 BIC = −79.7, fmm1 BIC = −51.4) (Fig. 6B). There was a significant negative relationship between $\log_{10}$ GSI and testis *amh* mRNA expression ($R^2 = 0.53$, $p < 0.0001$) (Fig. 7A) and a significant positive relationship between $\log_{10}$ GSI and testis *igf3* mRNA expression ($R^2 = 0.40$, $p < 0.0001$) (Fig. 7B).

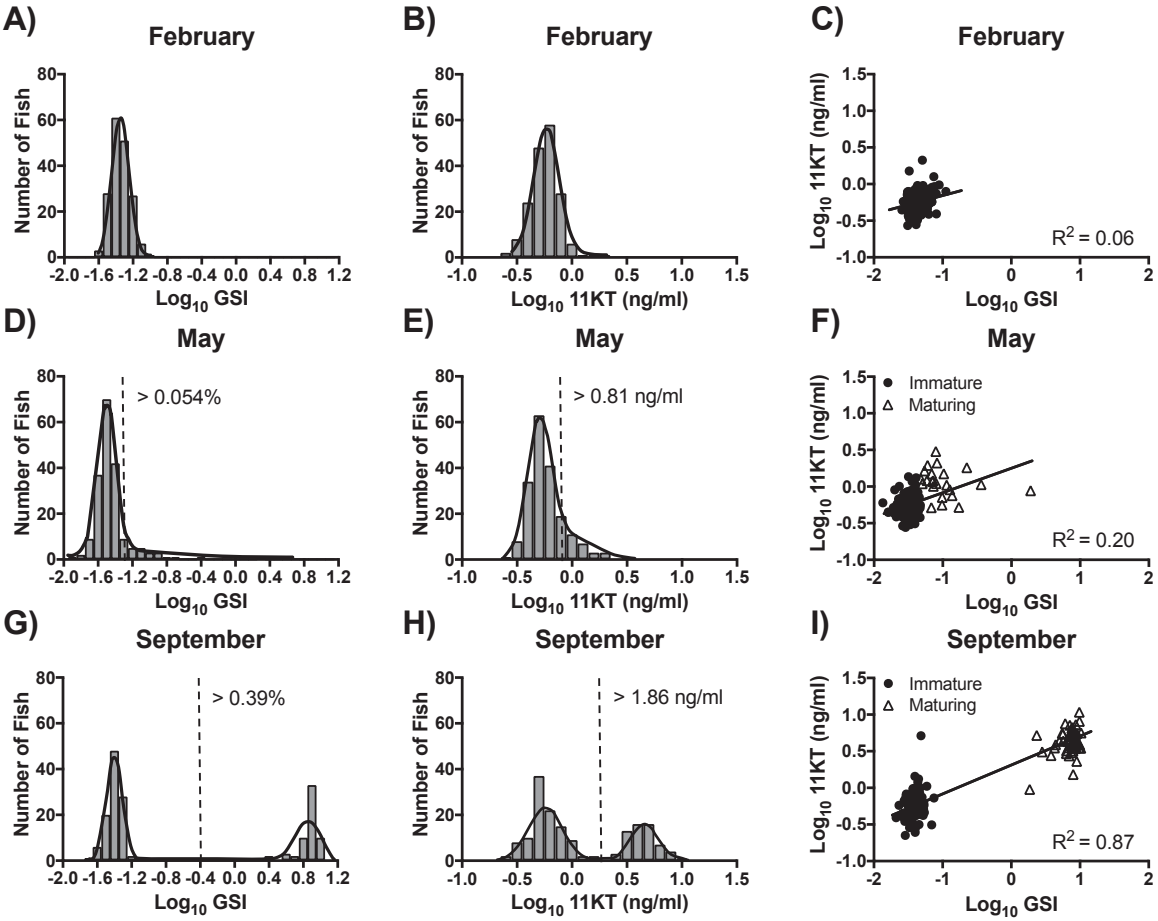

Fig. 3. Frequency distributions and linear regressions of male gonadosomatic index (GSI) and plasma 11-ketotestosterone (11KT) for fish sampled in February (A-C, N = 177), May (D-F, N = 191), and September (G-I, N = 167). Dashed lines on frequency distributions (A-B, D-E, and G-H) indicate the boundary between modes determined by a finite mixture model analysis. Samples with measurements greater than the dashed line value are considered maturing while samples with measurements less than the dashed line value are considered immature. In panel F, (•) represent fish classified histologically as immature (stage 0) and (Δ) represent fish classified histologically as maturing (stages 1–3). In panel I, (•) represent fish with $\log_{10}$ GSI < −0.4 which are likely immature and (Δ) represent fish with $\log_{10}$ GSI > −0.4 which are likely maturing.

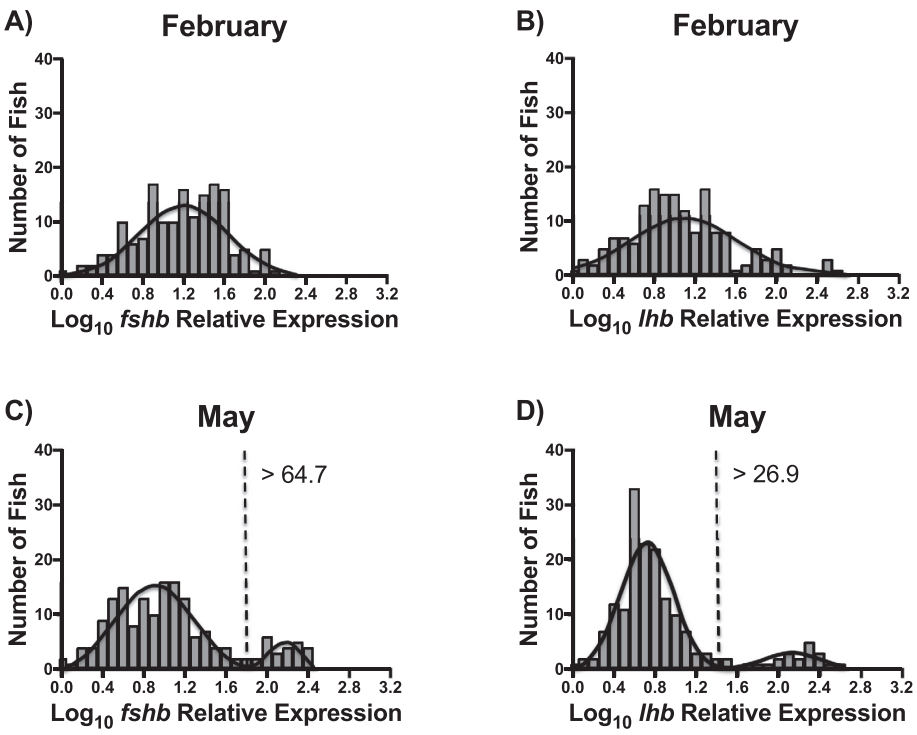

Fig. 4. Frequency distributions of relative mRNA expression levels for pituitary *fshb* and *lhb* of males sampled in February (A-B, N = 164) and May (C-D, N = 173). Dashed lines on May frequency distributions (C, D) indicate the boundary between modes determined by a finite mixture model analysis. Samples with measurements greater than the dashed line value are considered maturing while samples with measurements less than the dashed line value are considered immature.

3.5. Testis histology – May

Stage of spermatogenesis was determined by testis histology in May samples only because fish sampled at this time showed variation in reproductive state based on GSI, pituitary *fshb* and *lhb*, and testis *amh* and *igf3* gene expression. Comparisons between immature and maturing fish for all parameters measured in May are shown in Table 3.

3.6. Percent of maturing fish – September vs. May

In September, there were 61 out of 167 males in the upper modes of both the GSI and plasma 11KT frequency distributions. We found that the percent of maturing males ranged from 20 to 60% among the 10 tanks sampled (total males per tank ranged from n = 12–23, maturing males per tank ranged from n = 3–9). Overall, the percent of maturing males in September was 36.8% ± 3.59 (mean ± standard error). The percent of males predicted to be mature the following spring based on each parameter measured in May, as well as a combination of those parameters, are shown in Table 4. The best predictor of male maturation was a combination of GSI, plasma 11KT and pituitary *fshb* and *lhb* gene expression; however, this combination of factors only predicted that 21% of males would mature for the following spring spawning season. Adding measures of testis *amh* and *igf3* expression and examining testis histology did not increase the percent of males predicted to mature. The second-best combination of factors was GSI and plasma 11KT, which predicted that 19% of males would mature by the following spring. Finally, plasma 11KT alone predicted that 17% of the males would be mature the following spring.

4. Discussion

The aim of the present study was to determine if a suite of endocrine and molecular markers could be used to estimate the proportion of male Steelhead Trout initiating puberty by the time of smolt releases, approximately 10–12 months before the fish would be expected to fully mature and spawn in the subsequent spring. Our choice of reproductive parameters to monitor as indices of puberty onset was based on work on the hormonal regulation of spermatogenesis in fish (reviewed by Schulz et al., 2010). We found that yearling Skookumchuck River winter-run Steelhead Trout that had initiated puberty (were in at least stage 1 of spermatogenesis) by May, also had significantly higher GSI and plasma 11KT, elevated expression of pituitary *fshb* and *lhb* and testis *igf3*, and reduced expression of testis *amh*. Based on these criteria, the proportion of males initiating puberty in May was 21%. This was substantially lower than the estimate of 37% found in samples collected from the same group of fish in September based solely on GSI and plasma 11KT. The percent of maturing males varied among tanks (range of 20–60%), likely due to a low N value of mature males in each tank (range 3–9). Sampling a larger N throughout the experiment potentially would have reduced the variation in percent maturation among tanks found in September. Still, using a combination of GSI, plasma 11KT, and pituitary *fshb* and *lhb*, conservative estimates of the proportion of males initiating maturation were obtained. Even more conservative estimates were achieved using GSI and plasma 11KT together or by using plasma 11KT alone. Nonetheless, conservative estimates could be sufficient for comparing environmental, experimental treatment, or family effects on the proportion of Steelhead Trout males maturing in hatcheries at the time of smolt releases. Additionally, measurement of plasma 11KT does not require lethal sampling and might prove desirable to hatcheries that do not wish to kill their production fish. Even extremely conservative estimates of proportion of maturing males could still prove beneficial to hatcheries that wish to reduce production of non-migratory males.

Previous studies demonstrated that Steelhead Trout residualizing in natal streams are a mixture of fish that failed to migrate because they either did not smolt or were maturing males (Larsen et al., 2017; Sharpe et al., 2007; Tatara et al., 2019). This suggests that, at the time of smolt release, a proportion of male Steelhead Trout have sufficient endocrine signaling and testis development to inhibit downstream migratory behavior. The most definitive indicator that puberty is initiated in male fish is the onset of spermatogenesis, the process in which diploid spermatogonia proliferate and differentiate to form mature spermatozoa (for a review of fish spermatogenesis see Schulz et al., 2010). Briefly, spermatogenesis can be divided into three different phases: the mitotic or spermatogonial phase with different generations of spermatogonia (undifferentiated spermatogonia, including spermatogonial stem cells, and differentiating or differentiated spermatogonia); the

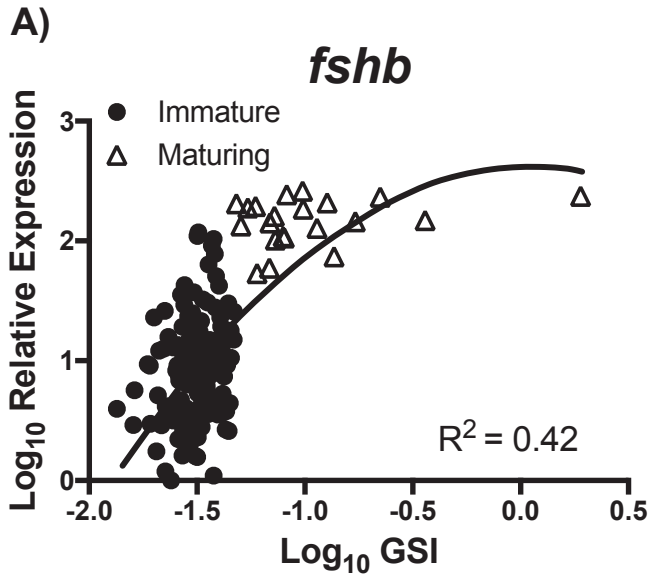

Fig. 5. Regressions of male gonadosomatic index (GSI) and relative expression of pituitary *fshb* (A) and *lhb* (B) mRNAs for fish sampled in May (N = 172). Immature fish (those classified histologically as stage 0) are represented by (•) and maturing fish (those classified histologically as stages 1–3) are represented by (Δ).

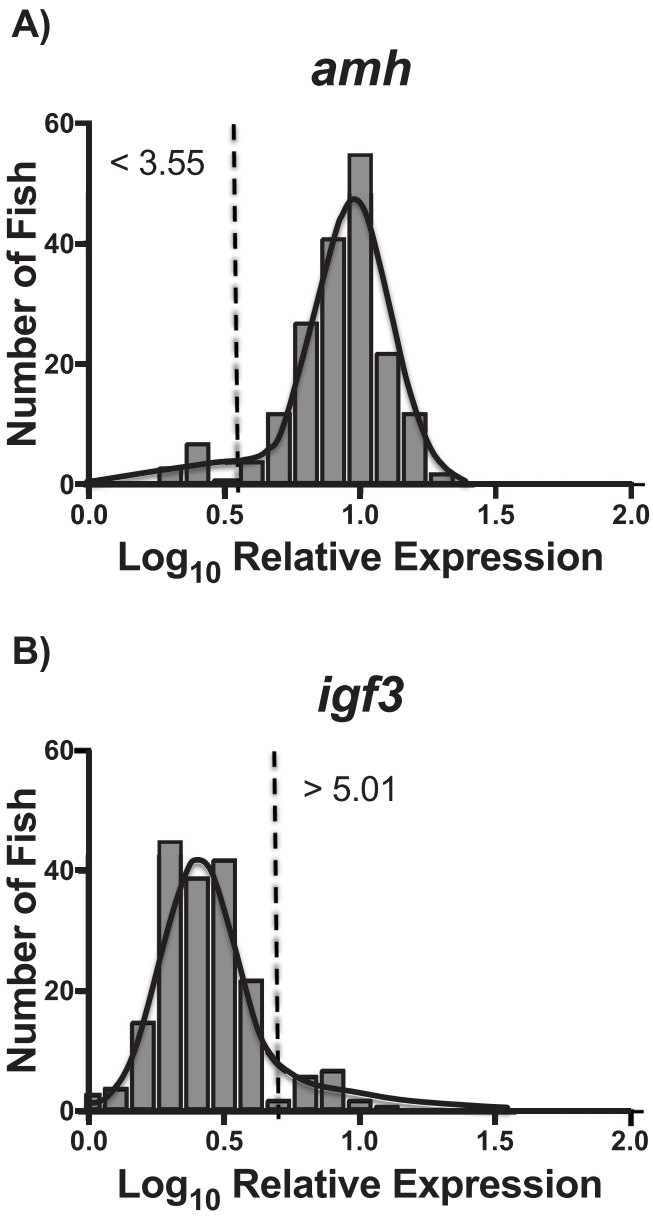

Fig. 6. Frequency distributions of relative mRNA expression levels for testis *amh* (A) and *igf3* (B) for males sampled in May (N = 187). Dashed lines indicate the boundary between modes determined by a finite mixture model analysis. In panel A, samples with measurements greater than the dashed line value are considered immature while samples with measurements less than the dashed line value are considered maturing. In panel B, samples with measurements greater than the dashed line value are considered maturing while samples with measurements less than the dashed line value are considered immature.

meiotic phase with primary and secondary spermatocytes; and the spermiogenic phase with haploid spermatids differentiating into motile spermatozoa with flagella. Visualizing the earliest phases of spermatogenesis is most commonly accomplished via testis histology because cell differentiation occurs prior to substantial changes in GSI. Using histology on a large scale is labor intensive and it is challenging to identify and quantify developmental stages of germ cells in large numbers of samples. Additionally, in this study we found that histology alone only identified 12% of males as maturing in May, a substantially smaller percentage than our best estimate utilizing other, less labor intensive, measures. Combining histological staging with other parameters measured also did not increase our estimates of the proportion of maturing males. For this reason, in this study we endeavored to identify the initial phases of spermatogenesis using different physiological markers of puberty.

Several studies have shown that initiation of spermatogenesis is regulated primarily by FSH in the pituitary gland, which in turn stimulates production of the major male androgen 11KT by the testis (Planas and Swanson, 1995). In both spring Chinook Salmon (Campbell et al., 2003) and Rainbow Trout (Gomez et al., 1999; Prat et al., 1996), plasma levels of FSH and 11KT increase during spermatogenesis while plasma levels of LH are low or undetectable until spermiogenesis and spermiation. These findings are consistent with studies in eel that have shown 11KT stimulates all stages of spermatogenesis and initiates the mitotic phase of spermatogenesis (Miura et al., 1991). FSH acts on the Sertoli cell to inhibit production of AMH which in turn inhibits spermatogonial proliferation (Miura et al., 2002). In addition, FSH acts on the Sertoli cell to increase IGF3 production which stimulates spermatogonial proliferation via IGF receptor signaling (Nobrega et al., 2015).

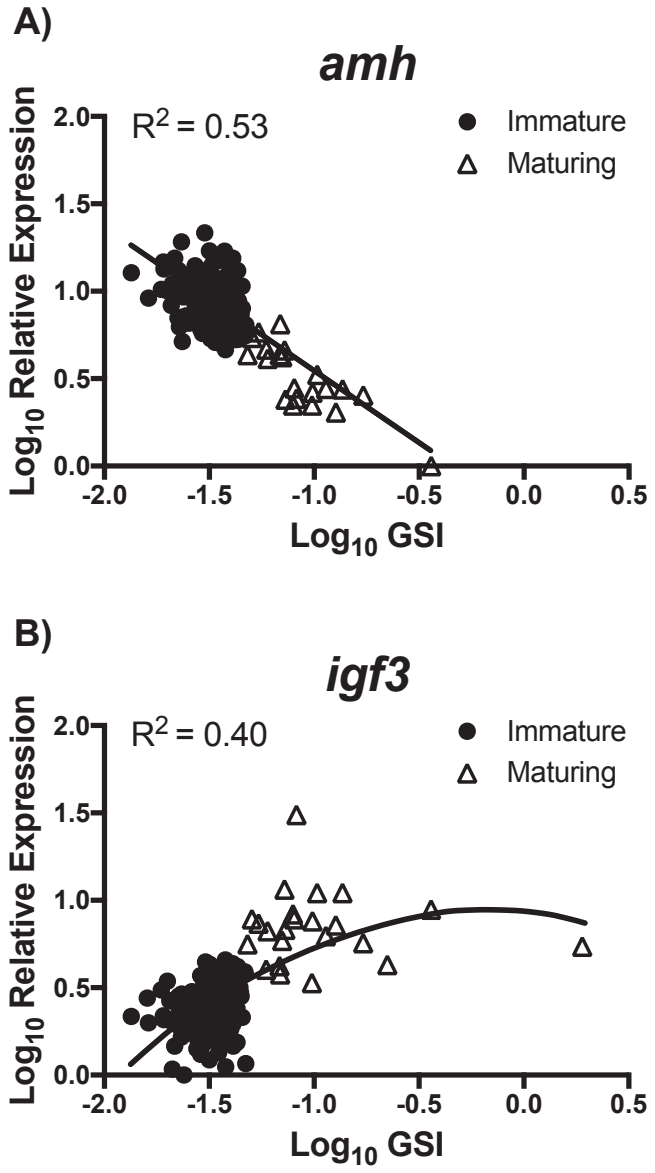

Fig. 7. Regressions of male gonadosomatic index (GSI) and relative expression of testis *amh* (A) and *igf3* (B) mRNAs for fish sampled in May (N = 186–188). Immature fish (those classified histologically as stage 0) are represented by (•) and maturing fish (those classified histologically as stages 1–3) are represented by (Δ).

Table 3
Body size, gonadosomatic index (GSI), plasma 11-ketotestosterone (11KT), pituitary *fshb* and *lhb*, and testis *amh* and *igf3* in male steelhead collected in May. "Immature" fish (N = 152–168) are those identified as stage 0 by histology and "Maturing" fish (N = 21–23) are those identified as stage 1–3 by histology. Data are mean ± standard errors. An asterisk indicates a significant difference between immature and maturing fish ($p < 0.05$).

Parameter Measured	Immature	Maturing
Fork Length (mm)	193.3 ± 1.34	191.7 ± 6.58
Body Weight (g)	76.63 ± 1.63	84.17 ± 9.13
GSI (%)	0.032 ± 0.001	0.182 ± 0.079*
11KT (ng/ml)	0.559 ± 0.014	1.238 ± 0.121*
pituitary *fshb*	13.77 ± 1.55	157.70 ± 13.40*
pituitary *lhb*	7.07 ± 0.97	163.80 ± 21.65*
testis *amh*	9.60 ± 0.23	3.48 ± 0.31*
testis *igf3*	2.53 ± 0.06	7.89 ± 1.15*

Previous studies indicated that once the mitotic phase of spermatogenesis is initiated *amh* mRNA declines and *igf3* mRNA increases (Nobrega et al., 2015; Skaar et al., 2011). These changes in *amh* and *igf3* mRNA take place prior to increases in mRNAs for steroidogenesis-related genes (e.g. steroidogenic acute regulatory protein, StAR) (Maugars and Schmitz, 2008a) that occur as 11KT levels increase (Kusakabe et al., 2006; Maugars and Schmitz, 2008a). In our study, we found pituitary *fshb* and *lhb* mRNA and plasma 11KT were all useful parameters in quantifying the proportion of males initiating maturation. However, we found that testis *amh* and *igf3* mRNA did not help to increase our estimates and it is possible that our samplings took place before vast changes in these genes were measurable.

Steelhead Trout initiating spermatogenesis by May were in a range of stages suggesting that the population was not synchronous. This finding was also reflected in the range of GSI, plasma 11KT, and pituitary and testis gene expression in May, and the GSI and plasma 11KT levels observed in maturing fish in September. In previous studies of spring Chinook Salmon, sampling occurred 6–9 months prior to spawning while the current study was approximately 10–12 months prior to spawning. Thus, the timing of puberty onset among the population of fish sampled appeared to vary considerably and some fish likely initiated puberty after the May sampling, resulting in underestimates of the proportion of precocious males. It should be noted that we had planned on sampling these fish in July, but we were unable to complete this objective. Unfortunately, shortly after sampling occurred in May, pipes providing water to our tanks became clogged with algae. The resulting lowered water flow, combined with fish density and warming seasonal temperatures, caused a mortality event of approximately 180 fish per tank (approximately 1800 fish total), likely due to hypoxia. It is possible that we would have been able to identify a higher proportion of males initiating maturation in July, compared to those in May, had we been able to analyze samples at that time point. Previous studies have demonstrated that male maturation varies over time (Kusakabe et al., 2006; Melo et al., 2014; Prat et al., 1996). Therefore, while all males synchronize their maturation status prior to spawning, some males may start the maturation process earlier or later than others. Our May sampling may have occurred prior to some males initiating the maturation process and, consequently, we were unable to capture that portion of the fish in our analyses.

A broader question arises as a result of this and previous work on puberty onset in anadromous salmon and Steelhead Trout. What is the relationship between seasonal timing of puberty onset relative to timing of upstream migration of adults and spawning? Upstream migration is inextricably linked to reproduction with the journey beginning in the ocean months before entry to freshwater habitats (Groot and Margolis, 1991; Quinn, 2005). The Steelhead Trout used for this study are a winter-run stock; mature adults return to natal rivers in late winter 1–2 months prior to spring spawning. In contrast, summer-run Steelhead Trout adults return to their natal rivers in summer approximately 9–10 months prior to spawning in spring of the following year. Interestingly, smoltification and outmigration of both life history variants occurs in the spring with precocious males residualizing in their natal stream (Larsen et al., 2017; Sharpe et al., 2007; Snow et al., 2013; Tatara et al., 2019). This suggests that, in the spring, puberty is sufficiently advanced in some fish to inhibit downstream migration. However, it is possible that the winter-run Steelhead Trout life-history variant initiates puberty slightly later in the spring than summer-run fish. If the seasonal timing of puberty onset is related to the timing of upstream migration, then the duration of spermatogenesis, and therefore maturation, may be more compressed in summer-run fish potentially due to warmer in-river temperatures. As such, the markers of puberty developed in this study may be able to identify a higher percentage of maturing males in a summer-run population since spermatogenesis may be more advanced in the spring in summer-run than winter-run fish.

Table 4
Estimates of percent of male steelhead predicted to be maturing by fall based on individual parameters measured, as well as combinations of measured parameters, in May. Percentages are based on counts of maturing males for each parameter, or a combination of parameters, divided by the total number of males for which that parameter(s) was measured (N = 173–191). Diagnostic accuracy is ranked from high percentage to low percentage, ranks in bold and underlined are the top three percentages utilizing the fewest parameters. Abbreviations are as follows: GSI, gonadosomatic index (%); 11KT, 11-ketotestosterone (ng/ml).

Parameter measured	Number of immature males	Number of maturing males	Predicted percent of males maturing	Rank of diagnostic accuracy
GSI	169	21	11%	6
11KT	159	32	17%	**3**
fshb	149	24	14%	4
lhb	150	23	13%	5
amh	175	12	6%	8
igf3	171	18	10%	7
histological stage	168	23	12%	5
GSI + 11KT	154	37	19%	**2**
GSI + 11KT + *fshb* + *lhb*	151	40	21%	**1**
GSI + 11KT + *amh* + *igf3*	154	37	19%	2
GSI + 11KT + *fshb* + *lhb* + *amh* + *igf3*	151	40	21%	1
GSI + 11KT + *fshb* + *lhb* + *amh* + *igf3* + histological stage	151	40	21%	1

5. Conclusions

This investigation successfully characterized the earliest stages of spermatogenesis in Steelhead Trout by measuring a suite of reproductive markers and demonstrated that one can obtain at least a conservative estimate of the proportion of male fish undergoing precocious maturation approximately one year prior to spawning. This analysis provides useful insights into the timing and relationship between previously established markers of salmonid maturation (GSI, plasma 11KT, FSH, LH) and more recent additions to this field of research (AMH and IGF3). However, our estimate of the proportion of fish undergoing precocious male maturation in May was substantially lower than our final estimate in September. This observation suggests that additional fish likely initiated maturation after our May sampling event, somewhat limiting our ability to accurately quantify rates of precocious male maturation prior to the release of smolts from a production Steelhead Trout hatchery. It should be emphasized that in May we successfully identified 57% of the male fish as initiating maturation nearly a year prior to spawning the following spring (21% initiating maturation in May vs. 37% maturing in September). This is not an insignificant achievement when compared to previous studies in Chinook Salmon (Larsen et al., 2004; Harstad et al., 2014) where maturing males were identified 6–7 months prior to spawning. Furthermore, for more controlled experiments or aquaculture applications, where fish may remain in culture for longer periods of time and are sampled later in the summer, these results provide great promise for identifying the proportion of fish initiating maturation many months in advance of completing testis growth and spawning. Additionally, it is conceivable that this type of study would work well in summer-run Steelhead Trout populations which potentially initiate spermatogenesis earlier in the season and have a more compressed period of maturation.

Declaration of Competing Interest

None. Use of trade names does not imply endorsement by the National Marine Fisheries Service, Department of Commerce.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.05.010.

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