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Accuracy of histology, endoscopy, ultrasonography, and plasma sex steroids in describing the population reproductive structure of hatchery-origin and wild white sturgeon

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Abstract

Hatchery-origin white sturgeon *Acipenser transmontanus* in the lower Columbia River, Canada are approaching puberty, and describing the reproductive structure of the population is critical to determine if they are capable of contributing to spawning events in the wild, a key management uncertainty. Few studies have compared the accuracy of available tools (histology, ultrasound, endoscopy, and plasma sex steroids) used to assign sex and stage of maturity within the same population of prepubertal and post-pubertal sturgeon. Population reproductive structure was described using these tools in 332 hatchery-origin and 75 wild individuals over 2 years (2017 and 2018). True sex was determined using histological analysis of gonadal tissue, which is 100% accurate at assigning sex and stage of maturity in fish when germ cells are present in the biopsy. All hatchery-origin fish assessed had not reached puberty and were pre-meiotic males (n = 158) or pre-vitellogenic females (n = 174). Assignment of true sex using histology was 97% in hatchery-origin and 94% in wild fish as several biopsies did not contain germ cells. Fish with gonadal biopsies that did not contain germ cells and intersex fish (n = 3) were not included in further analyses of other tools. Accuracy in assigning sex to both the hatchery-origin (98%) and wild (100%) fish was highest using endoscopy (an otoscope). The other tools evaluated were less accurate, with 69% accuracy in hatchery-origin and 74% accuracy in wild fish for plasma sex steroids and 57% accuracy in hatchery-origin and 70% accuracy in wild fish for ultrasonography. Based on these results, endoscopy was the most reliable tool for assigning sex in both prepubertal and post-pubertal fish and can be easily complimented with histology when determining stage of maturity or describing population reproductive structure.

KEYWORDS

endoscopy, hatchery-origin, histology, reproductive structure, sex assignment, steroids, sturgeon, ultrasound

1 | INTRODUCTION

Understanding the reproductive dynamics of a population is essential to the successful management of fishes (Jakobsen et al., 2016), and reproductive indices (e.g., sex ratio, age and size at puberty or first spawning, population reproductive structure, fecundity, and spawning periodicity) are used to describe the reproductive dynamics of a population. Specifically, population reproductive structure is defined as the proportion of the population in each stage of the reproductive cycle. The proportion of females and males at each stage of maturity can be used as a metric to evaluate the reproductive potential of a population and explore future population dynamics (Lowerre-Barbieri et al., 2011; Worm et al., 2009). In populations being supplemented by conservation aquaculture programs, assessing the proportion of the population that is prepubertal and post-pubertal allows for determination of age and size at puberty and if the hatchery-origin population is contributing to wild spawning events.

Sturgeons and paddlefish are not sexually dimorphic (Billard & Lecointre, 2001; Haxton et al., 2016), therefore describing the population reproductive structure is potentially time consuming and costly. Furthermore, collecting biological samples may be difficult if the population is threatened or endangered (Webb et al., 2019). However, numerous tools (e.g., gonadal biopsy, endoscopy, ultrasonography, and plasma sex steroid analysis) have been developed to assign sex and stage of maturity in sturgeons by non-invasive or minimally invasive techniques (e.g., Du et al., 2017; Feist et al., 2004; Moghim et al., 2002; Munhofen et al., 2014; Webb et al., 2019). Training requirements can be quite specific for certain tools (e.g., histology; Webb et al., 2019) and the applicability or accuracy may vary depending on the age or size of fish being studied. Reproductive indices have been increasingly incorporated into the management of conservation and recovery programs for sturgeons, in particular for populations that have been supplemented with hatchery-origin fish as many programs have been underway for decades. Despite the need to understand reproductive development of hatchery-origin sturgeon in the wild, comparative field studies to describe the efficacy of various tools have been limited.

White sturgeon *Acipenser transmontanus* in the lower Columbia River (LCR), Canada, were listed as endangered in 2006 under the Species at Risk Act. While it is a transboundary population, the section of the population in the United States is not listed. Wild adults in the population continue to spawn; however, age-0 individuals are not surviving to reproduce, and this recruitment failure threatens the persistence of the entire population (Hildebrand & Parsley, 2013). To address recruitment failure, conservation aquaculture was implemented in 2001 in an effort to restore natural age class structure and has been successful, with juveniles at large in the population from 19 age classes (Crossman & Korman, 2021). While information on post-release survival (BC Hydro, 2018) has been used to adaptively manage the conservation aquaculture program, it is uncertain if older or larger hatchery-origin white sturgeon have reached puberty and are introgressing with wild spawners.

The goal of this study was to determine if the hatchery-origin white sturgeon in the LCR have reached puberty. Puberty is the developmental period during which a fish becomes capable of reproducing for the first time (Taranger et al., 2010), and initiation of puberty in this study was identified by the onset of vitellogenesis and/or spermiogenesis which includes a concomitant increase in plasma sex steroids (testosterone [T] and estradiol-17β [E2] in females and T in males; Webb & Doroshov, 2011). To accomplish this goal, we described the population reproductive structure using available tools to assign sex and stage of maturity (gonadal biopsy analyzed histologically, ultrasonography, endoscopy, and plasma sex steroids). Accuracy in assigning sex using each tool was determined in hatchery-origin white sturgeon and a small number of the existing wild white sturgeon. The wild fish enabled a comparison of accuracy in assigning sex between prepubertal and post-pubertal animals. Results from this study will inform the most effective tool for describing the reproductive structure of sturgeon populations being supplemented with hatchery-origin progeny.

2 | STUDY AREA

The white sturgeon population resides in the Transboundary Reach of the Columbia River. Sampling was conducted in both Canada and the United States, though the primary study site was located in Canada, throughout a 57-km reach of the LCR, between Hugh L. Keenleyside Dam (river kilometer [rkm] 0.0) and the United States-Canada border (rkm 57) in British Columbia, Canada (Figure 1). Sampling was divided among three zones, which were delineated by differences in discharge, available habitat, and previous data on differences in growth (BC Hydro, 2018) and diet (Crossman et al., 2016). White Sturgeon abundance was highest in zone 1 (BC Hydro, 2018); therefore, in an attempt to equalize the distribution of samples among zones, the zone length (in rkm(s)) was greater in zones 2 and 3. Zone 1 was 11-rkm(s), and zones 2 and 3 were each 23-rkm(s). A small number of hatchery-origin white sturgeon were also collected from the upper Columbia River in Lake Roosevelt, which is located in northeast Washington, USA, for the homogeneity component of the study (see section 3.3.6). White sturgeon from Lake Roosevelt were collected during a larger on-going population assessment program conducted by the Confederated Tribes of the Colville Reservation in spring and fall of 2016 and fall of 2018 (Howell & McLellan, 2018).

3 | METHODS

3.1 | Sampling design

Sampling was conducted in 2017–2018, with capture events in the spring and fall of each year. The population of LCR white sturgeon has high site fidelity (BC Hydro, 2016). Therefore, the capture effort was spatially balanced throughout the fishable areas within the river, and all habitat types were included. Locations of sampling sites were

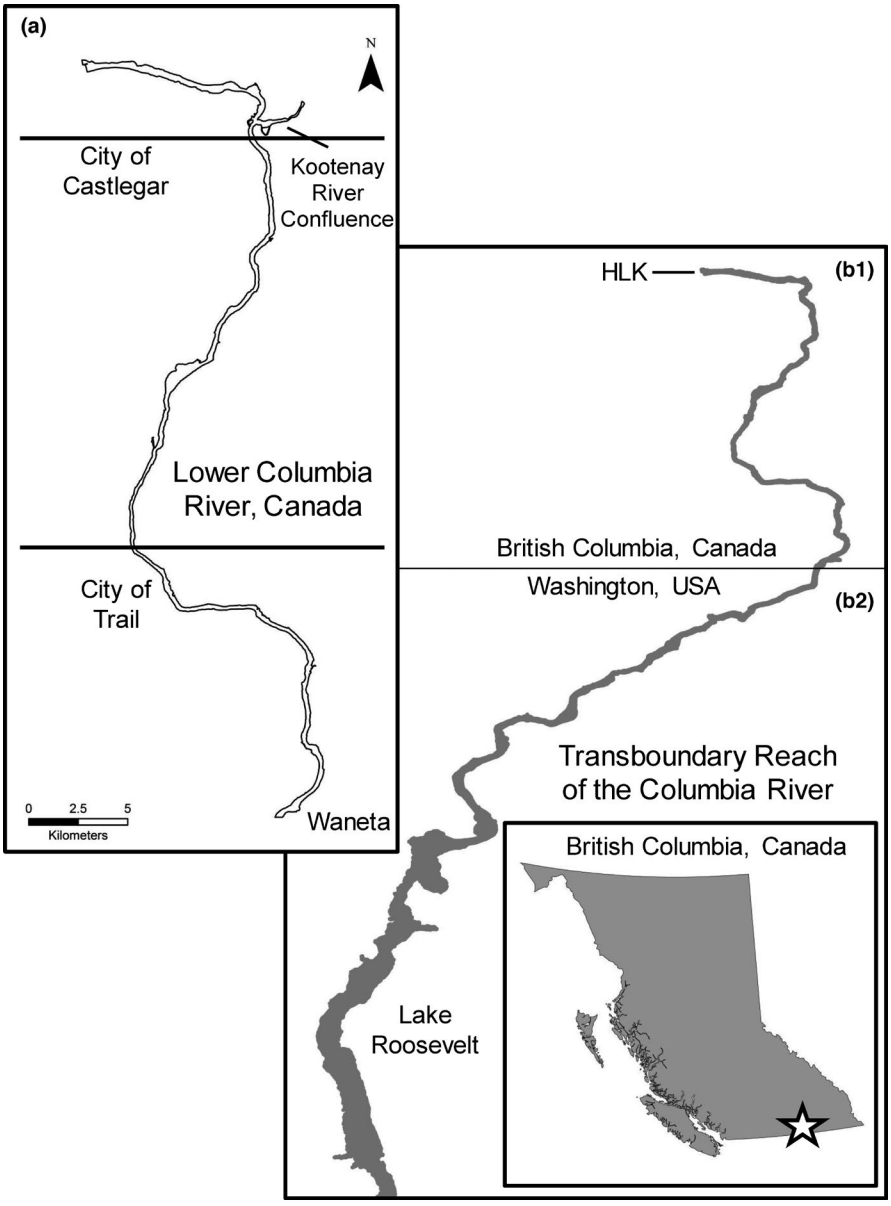

FIGURE 1 Map depicting the study site, which was 57 river kilometers (rkm) of the lower Columbia River (LCR), Canada, between Hugh L. Keenleyside Dam (HLK) and the international border. Panel (a) is a zoomed in view of the study site (b.1) within the transboundary reach of the Columbia River which includes Lake Roosevelt in Washington (b.2). The black horizontal lines represent the different zones within the LCR. Zone 1 was 11-rkm and zones 2 and 3 were 23-rkm each. Figure modified from BC Hydro (2016)

randomly assigned with 24 sites in zone 1 and 48 sites in each of zones 2 and 3. Capture probability was assumed to be equal among all sampling zones. Sampling began at the most downstream location in zone 3 and progressed upstream until all sites were sampled.

Juvenile white sturgeon from the conservation aquaculture program were marked individually with passive integrated transponder (PIT) tags prior to release, and releases occurred annually from 2001 until the time of this study. In the spring of 2017, up to six hatchery-origin fish from the oldest 6-year classes (2001–2007) and six wild fish were selected for the study from each zone. We balanced samples according to year class, rather than age, as the oldest year classes are reliably captured in the highest numbers during sampling (Crossman & Korman, 2021). Thus, the ages of white sturgeon were 10–16 in 2017 and 11–17 in 2018. The sampling design was adjusted prior to the start of fall 2017 in an effort to decrease the number of minor surgical biopsies conducted for the initial sampling design. The adjusted design remained in place for the spring and fall of 2018, and in each sampling zone, up to four hatchery-origin fish per year class and five wild fish were included.

3.2 | Fish capture

Sampling gear was standardized among all sessions. White sturgeon were captured using set lines baited with pickled squid (*Loligo* spp.) with one line deployed at each sampling location for a total of 120 set lines. Set lines were deployed for 24 h and were approximately 54 m in length, and 12 circle halibut barbless hooks of four different sizes (14/0, 16/0, 18/0, and 20/0) were attached to the mainline at 6-m intervals. Once captured, fish were scanned for a PIT tag, and only fish with confirmed year classes were included in the study. All captured fish were measured for fork length (to the nearest half cm) and body weight (±0.02 kg). Fish were not anesthetized (see Webb et al., 2019).

3.3 | Assignment of sex and stage of maturity

The tools are described in the same order as they were applied in the field. Ultrasonography and endoscopy were alternately conducted on fish to assign sex and ensure that accuracy for each tool could be determined without bias. For wild fish in the fall of 2017 and 2018, only ultrasonography was used to assign sex as endoscopy has historically and successfully been used to assign sex in the wild population.

3.3.1 | Plasma sex steroids

Blood (2 ml) was collected from the caudal vasculature using a 2-ml syringe, placed in a heparinized vacutainer, and centrifuged at 1228 *g* (relative centrifugal force) for 5 min. Plasma was frozen and stored at −80°C until laboratory analysis. Steroids were extracted from the plasma following the method of Fitzpatrick et al. (1987). Steroids (T and E2) were measured by radioimmunoassay (RIA) as described by Fitzpatrick et al. (1987) and modified by Feist et al. (1990). Recovery efficiencies were 90%–98% for T and 73%–93% for E2. All steroid assay results were corrected for recovery. Quantified plasma sex steroid concentrations below the minimum quantifiable concentration (MQC) of the RIA were assigned the MQC (0.16–0.38 ng/ml for T and 0.10–0.21 ng/ml for E2). Non-detectable plasma sex steroid concentrations (i.e., not quantifiable) were assigned half of the MQC for statistical purposes (0.08–0.19 ng/ml for T and 0.05–0.11 ng/ml for E2; Croghan & Egeghy, 2003). The intra- and inter-assay coefficients of variation for all assays were <10%.

3.3.2 | Ultrasonography

An ultrasound (Sonosite Edge II with a 6–15 mHz linear transducer) was used to capture images of the gonad which enabled the assignment of sex based on differences in echogenicity, echotexture, and uniformity of gonadal tissue. The Small Parts exam type was used with the optimization set to General and the scanning depth set between 4.0 and 6.0 cm. A black sheet was draped over the sonographer to create shade to adequately view the ultrasound screen. Fish were laid dorsally in the stretcher, and the ultrasound transducer was held (1) posterior to the urogenital pore and perpendicular to the ventral scutes between the anal fin and the second to third ventral scutes and (2) laterally and parallel to the scutes (longitudinal plane), approximately two to four scutes anterior of the urogenital pore. All sonograms were archived.

Using ultrasonography, fish were assigned as female based on the appearance of fine-grains and an irregular shape of the gonad without a clearly defined tunica or the presence of ovarian follicles (Chebanov & Galich, 2009). Fish were assigned as male based on smooth margins of the gonad and a bright hyperechoic tunica (Chebanov & Galich, 2009). Sex was assigned blindly by each project member. Field assignment of sex for an individual using ultrasonography was based on the majority assignment of the project members (n = 3). The accuracy of using ultrasonography to assign sex was determined by comparing the sex assignment by ultrasonography to the true sex determined by gonadal histology (section 3.3.5).

3.3.3 | Endoscopy

Endoscopy using an otoscope (Welch Allyn, 3.5 V) was used to macroscopically identify the gonad which enabled the assignment of sex based on morphological characteristics. The otoscope was inserted into the body cavity through an abdominal incision. The incision site was approximately 1–3 cm off the midline between the third and fourth scutes, anterior to the urogenital pore, and approximately 1- to 2-cm long. The otoscope specula was cleaned and disinfected between each fish with 90% ethanol.

Sex and stage of maturity were assigned based on the stage descriptions in Table 1 (Webb et al., 2019). Fish were assigned as female based on the presence of ovigerous folds, oocytes, or ovarian follicles and assigned as male based on the presence of testicular lobes. Sex was assigned blindly by each project member, and field assignment of sex for an individual was based on the majority assignment of the project members (n = 3). The accuracy of using endoscopy to assign sex was determined by comparing the sex assignment by endoscopy to the true sex determined by gonadal histology. Images were captured and archived using a Vividia 2.0 MP handheld USB digital endoscope (Oasis Scientific, Inc.).

3.3.4 | Gonadal biopsy

Following visual assignment of sex using endoscopy, gonadal tissue (5 mm^3) was collected through the otoscope specula using Miltex biopsy forceps and stored in 10% phosphate buffered formalin. Incisions were closed with 2–3 sutures using polydioxanone violet monofilament suture. All fish were released alive.

3.3.5 | Assignment of true sex

True sex and stage of maturity were determined in all fish using histological analysis of gonadal tissue and assigned based on descriptions in Table 1. Accuracy of histological analysis was dependent on collection of germ cells within the biopsy. Fixed gonadal tissue was processed according to Webb and Erickson (2007). Tissue from hatchery-origin fish was stained with hematoxylin and eosin, and tissue from wild fish was stained with periodic acid-Schiff to assist with identification of post-ovulatory follicles (Luna, 1968). Slides were examined using a compound scope (Leica DM 2000, 50–400×; Leica Biosystems, Inc.). Sex and stage of maturity for all fish in the study was assigned by two readers independently. If sex or stage of maturity differed between readers, the slide was reviewed by a third

TABLE 1 Histological descriptions of stages of maturity in sturgeon female and male gonadal development

Female developmental stages		Description
Differentiation	1	Clusters of oogonia and potentially a few very small oocytes just beginning the endogenous growth phase
Pre-vitellogenic	2	Perinucleolar oocytes in the endogenous growth phase. Follicular epithelium (granulosa) in the larger oocytes begins mitotic proliferation, and the outer follicular layer (theca) has some vascularization
Early to mid-vitellogenic	3	Differentiation of the zona radiata and the presence of yolk platelets in the cytoplasm. The granulosa cells increase in thickness and become cuboidal. Density and size of yolk platelets increases. Germinal vesicle is centrally located
Late vitellogenic	4	Melanin pigment deposited under the oolemma. Follicular layers and three layers of the chorion are fully differentiated. The nucleus begins to move off-center toward the animal pole
Post-vitellogenic/Ripe	5	Nucleus displaced to animal pole. Oocyte has polarized structure with animal hemisphere containing the bulk of the cytoplasm and small, round yolk platelets and lipid inclusions, while vegetal hemisphere contains large, oval-shaped yolk platelets and numerous large lipid inclusions
Oocyte maturation and ovulation	6	Germinal vesicle breakdown and ovulation have occurred
Post-ovulatory	7	Ovaries contain numerous postovulatory follicles and the next generation of oocytes similar to Stage 2 and sometimes Stage 3. The often reddish appearance of ovarian tissue is a result of vascularization for weeks after spawning.
Male developmental stages		**Description**
Differentiation	1	Clusters of primary spermatogonia
Pre-meiotic	2	Spermatogonia undergoing mitosis
Onset of meiosis	3	Spermatogonia (≈50%) and spermatocytes
Meiotic	4	Majority of cysts contain spermatocytes and spermatids, with <25% of cysts containing spermatogonia
Mature	5	Cysts and ducts contain almost all spermatozoa, but animal is not actively spermiating
Spermiation	6	Spermatozoa in all cysts and ducts, and animal is actively spermiating
Post-spermiation	7	Residual spermatozoa in regressed testicular cysts

Source: Reproduced from Webb et al. (2019).

reader. Furthermore, if a slide did not contain germ cells, the slide was sent to the third reader for confirmation, and the sex and stage of maturity was classified as unknown.

3.3.6 | Gonadal homogeneity

Gonadal homogeneity was assessed to ensure that collection of a gonadal biopsy represented the sex and stage of maturity between and across gonadal lobes within an individual. Gonadal biopsies were collected from a subsample of hatchery-origin white sturgeon in the LCR in 2017 ($n = 9$) as well as Lake Roosevelt ($n = 27$) as a portion of individuals within Lake Roosevelt have begun to initiate puberty (Webb et al., 2016). Samples from Lake Roosevelt were collected during a prior study in 2016 and during the 2018 study year. The samples from Lake Roosevelt in 2016 consisted of a 5 × 8-cm section of gonadal tissue (only one lobe sampled) from 24 hatchery-origin male white sturgeon. From these samples, three gonadal tissue biopsies (Miltex biopsy cup) were randomly collected. Each biopsy was labelled with fish ID, stored in 10% phosphate buffered formalin, and processed histologically.

For samples in 2017 and 2018, we attempted to replicate the approach described for collection of the gonadal biopsy in the field. Fish were euthanized following methods agreed upon with First Nations and regulatory agencies in both countries. An incision was made from the urogenital pore to the pectoral girdle and both lobes of the gonad were excised ($n = 9$ fish in 2017, 5 males and 4 females; $n = 3$ fish in 2018, 3 males and 0 females). A total of 20 biopsies ($n = 10$ per lobe) were collected (anterior to posterior) in the 2017 fish using a Miltex biopsy cup. Due to significant processing time, sample collection was reduced in 2018 to six biopsies ($n = 3$ per lobe; anterior, middle, posterior). Each biopsy was processed as described above.

Gonadal development was classified as either homogeneous or non-homogenous. The natural progression of spermatogenesis involves the proliferation and differentiation of spermatogonia

to spermatocytes to spermatids to spermatozoa (Doroshov et al., 1997). Testicular cysts within homogeneous gonads contain germ cells in multiple stages of development, yet the stages follow the natural progression of germ cell maturation (i.e., gonadal tissue contained spermatogonia only or spermatogonia and spermatocytes). In non-homogeneous gonadal development, testicular cysts contained germ cells in stages of maturity that did not develop within the natural progression of spermatogenesis (i.e., gonadal tissue contained predominately testicular cysts with spermatogonia with few cysts containing spermatids or spermatozoa, and spermatocytes were missing). The cysts containing spermatids and spermatozoa were well in advance of the predominant stage of maturity (spermatogonia), and the gonad was classified as non-homogeneous with precocious cysts.

3.4 | Analysis

The length–weight relationship of fish selected for the study was compared to that of all fish captured to determine if they were representative of the population. All data used for statistical analyses were visually assessed for normality using histograms, side-by-side boxplots, and diagnostic plots. Fork length and body weight were normally distributed, and one extreme outlier was identified and removed prior to statistical analyses. One-way analysis of variance (ANOVA) was used to determine if males and females differed by size (fork length and body weight), and this was repeated separately for hatchery-origin and wild sturgeon.

Percent accuracy of collecting a gonadal biopsy containing germ cells was determined as the number of biopsies collected with germ cells divided by the total number of biopsies collected. Percent accuracy of correctly assigning sex to each fish using ultrasonography and endoscopy was determined as the number of fish assigned to a sex (female or male; intersex fish not included) using each tool divided by the true number of fish (of each sex) determined histologically.

Statistical tests were used to determine if differences existed in the size of the fish assessed and plasma sex steroid concentrations. Plasma T and E2 concentrations collected in 2017 and 2018 were not normally distributed. Log_{10} transformation normalized T data but not E2 data, therefore non-parametric statistics were used for E2. A paired *t*-test was used to determine if mean log_{10} T concentrations differed by sex within the hatchery-origin population. A one-way ANOVA and a Tukey multiple mean comparisons test were used to determine if log_{10} differed by sampling season (season and year). A Kruskal–Wallis test was used to determine if E2 concentrations differed by sex within the hatchery-origin population. When assessing the difference in E2 concentrations by sampling season, a Kruskal–Wallis test with a Bonferroni correction was used. When assessing the wild population, a one-way ANOVA and a Tukey multiple comparisons test were used to determine if log_{10} T differed among stages of maturity within each sex. A Kruskal–Wallis test with a Bonferroni correction was conducted to assess if E2 differed among stages of maturity within each sex. A single atretic wild female (Stage 8) was not included in these statistical analyses. All data are presented as means (95% confidence intervals) unless otherwise specified. The accepted significance level for all tests was $\alpha = .05$ unless adjusted by the Bonferroni procedure. All statistical tests were completed using R software (R Core Team, 2019; version 3.6.1).

Quadratic discriminant function analysis (DFA) was used to determine the accuracy in assigning sex to the hatchery-origin and wild white sturgeon using plasma sex steroids (log_{10} transformed) and biological data (fork length and body weight) as described in Webb et al. (2002) and Malekzadeh Viayeh et al. (2006). Plasma E2 was not used in the analysis of hatchery-origin fish as concentrations were not detected. All DFA analyses were conducted using Statistical Analysis Systems for Windows, release 9.4M6 (SAS Institute).

4 | RESULTS

4.1 | Fish size

Fork length did not differ between hatchery-origin males and females [$F_{(1, 330)} = 2.02$, $p = .16$], with males averaging 100.5 cm (98.0–103.0 cm) and females averaging 98.5 cm (97.0–100.0 cm). Similarly, body weight did not differ between sexes [$F_{(1, 330)} = 1.76$, $p = .19$], with males averaging 6.99 kg (6.50–7.48 kg) and females averaging 6.52 kg (6.04–7.00 kg). Fish included in the study were representative of the length–weight relationship for the population (Figure 2), and size increased slightly with increasing age for both females and males (Table 2). Within the wild fish, average fork length [$F_{(1, 74)} = 5.71$, $p = .02$] differed between females (184.5 cm [177.0–192.0 cm; $n = 75$]) and males (171.0 cm [164.0–177.3 cm; $n = 25$]), with females being slightly longer (on average 14 cm; Table 2). Females and males also differed in body weight [$F_{(1, 74)} = 12.94$, $p \leq .001$]. Females were heavier (on average 15 kg; Table 2) and averaged 52.0 kg (46.5–57.1 kg) compared to males that averaged 36.5 kg (31.5–41.6 kg).

4.2 | Plasma sex steroids

Blood plasma sex steroids were analyzed by RIA in 331 hatchery-origin and 74 wild white sturgeon with histologically confirmed sex and stage of maturity. Plasma T concentrations differed by sampling season in hatchery-origin females [$F_{(3, 170)} = 23.29$, $p \leq .001$] and in hatchery-origin males [$F_{(3, 153)} = 8.98$, $p \leq .001$]. Similarly, plasma E2 concentrations differed by season in hatchery-origin females ($\chi^2 = 37.0$, df = 3, $p \leq .001$) and hatchery-origin males ($\chi^2 = 30.86$, df = 3, $p \leq .001$). However, the differences in plasma sex steroids between spring and fall for each year within the hatchery-origin population were not biologically relevant (<1 ng/ml), therefore, fish from all sampling seasons were combined for steroid analyses. Plasma T concentrations differed between hatchery-origin females and males ($T = -8.60$, df = 329, $p \leq .001$), with T concentrations higher in males compared to females (Table 3). Plasma E2 concentrations were not different between hatchery-origin females and males ($\chi^2 = 0.37$,

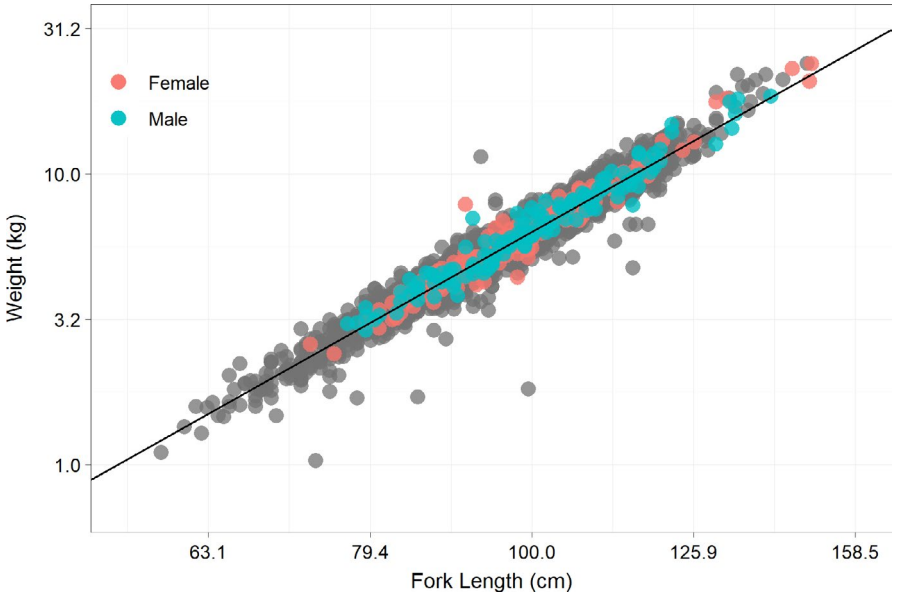

FIGURE 2 Weight–length relationship of all hatchery-origin white sturgeon ($n = 2738$) captured in the population assessment of the lower Columbia River (LCR), Canada (2013–2018). Fish assigned as female or male using histology are delineated by colour while grey circles were fish captured without sex assigned. The relationship is plotted on a log scale, but axis labels show non-transformed units

TABLE 2 Mean fork length (cm) at age (years) for female and male hatchery-origin and wild white sturgeon in the lower Columbia River, Canada

Age (years)	Mean female fork length (SD)	Mean male fork length (SD)
Hatchery-origin		
10 ($n = 17$)	89.6 (15.4) ($n = 8$)	88.7 (7.9) ($n = 9$)
11 ($n = 41$)	92.1 (15.0) ($n = 21$)	92.0 (11.0) ($n = 20$)
12 ($n = 46$)	92.4 (11.3) ($n = 25$)	101.8 (15.5) ($n = 21$)
13 ($n = 48$)	94.6 (12.3) ($n = 22$)	99.5 (13.6) ($n = 26$)
14 ($n = 42$)	100.2 (12.2) ($n = 23$)	103.6 (13.2) ($n = 19$)
15 ($n = 52$)	102.1 (7.9) ($n = 31$)	104.1 (8.7) ($n = 21$)
16 ($n = 64$)	105.6 (11.6) ($n = 34$)	101.4 (13.3) ($n = 30$)
17 ($n = 21$)	103.2 (12.5) ($n = 10$)	111.4 (15.0) ($n = 11$)
Wild ($n = 75$)	184.5 (26.4) ($n = 50$)	170.7 (17.0) ($n = 25$)

Note: Age data was not available for wild fish. Sample sizes (n) are in parentheses. One extreme outlier was identified and removed prior to analyses.

Abbreviation: SD, standard deviation.

TABLE 3 Plasma testosterone (T) and estradiol-17β (E2) concentrations (ng/ml) in hatchery-origin white sturgeon in the lower Columbia River, Canada

Sex	Stage of maturity	T (ng/ml)	E2 (ng/ml)
Females	Pre-vitellogenic ($n = 174$)	0.58 ± 0.07^a (0.10–9.99)	$0.10 + 0.01^a$ (0.05–0.78)
Males	Pre-meiotic ($n = 157$)	1.51 ± 0.13^b (0.12–7.50)	0.10 ± 0.004^a (0.05–0.51)

Note: Data are mean (±SEM), ranges (min. and max.), and sample size (n) in parentheses. Different letters denote statistical differences between females and males within a steroid. Plasma sex steroid concentrations below the MQC of the radioimmunoassay were assigned the MQC (0.16–0.38 ng/ml for T and 0.10–0.21 ng/ml for E2). Non-detectable plasma sex steroid concentrations were assigned half of the MQC (0.08–0.19 ng/ml for T and 0.05–0.11 ng/ml for E2).

Abbreviations: MQC, minimum quantifiable concentration; SEM, standard error of the mean.

df = 1, $p = .54$; Table 3). Plasma T was the best predictor of sex using DFA. The use of plasma T resulted in the correct assignment of sex in 78% of the females and 60% of the males (Table 4). Overall, 69% of hatchery-origin sturgeon were correctly classified by sex.

Plasma T concentrations differed by stage of maturity in wild white sturgeon females [$F_{(2, 45)} = 29.69$, $p \leq .001$] and males [$F_{(2, 22)} = 22.68$, $p \leq .001$; Table 5]. Plasma T was lower in pre-meiotic males (Stage 2) compared to meiotic (Stage 4) and mature (Stage 5) wild males (Table 5). Within females, T concentrations were different (Table 5) among pre-vitellogenic (Stage 2), early to mid-vitellogenic (Stage 3), and late vitellogenic females (Stage 4). Plasma E2 concentrations differed in wild early to mid-vitellogenic (Stage 3) and late vitellogenic females (Stage 4) as compared to pre-vitellogenic females (Stage 2) ($\chi^2 = 27.04$, df = 2, $p = .001$; Table 5). All E2 concentrations were non-detectable in wild males. Though steroid concentrations differed by sex and stage of maturity, the DFA examining the use of steroids to assign sex in these fish resulted in plasma T, E2, body weight, and length as the best predictors resulting in correct assignment in 51% of the females and 96% of the males (Table 4). Overall, 74% of wild sturgeon were correctly classified.

TABLE 4 Accuracy in assigning sex to the hatchery-origin and wild white sturgeon in the lower Columbia River, Canada using endoscopy, ultrasonography, and measurement of plasma sex steroids

Tool	Origin	Females	Males	Overall
Endoscopy	Hatchery	97 (89%–100%) (n = 65)	100 (95%–100%) (n = 66)	98 (95%–100%) (n = 131)
	Wild	100 (48%–100%) (n = 5)	100 (16%–100%) (n = 2)	100 (60%–100%) (n = 7)
Ultrasonography	Hatchery	74 (63%–83%) (n = 77)	39 (28%–51%) (n = 72)	57 (49%–65%) (n = 149)
	Wild	82 (65%–92%) (n = 38)	44 (22%–69%) (n = 18)	70 (56%–81%) (n = 56)
Plasma sex steroids	Hatchery	78 (n = 174)	60 (n = 157)	69 (n = 331)
	Wild	51 (n = 49)	96 (n = 25)	74 (n = 74)

Note: Endoscopy and ultrasonography are expressed as percent accuracy (95% CI). Accuracy using plasma sex steroids was estimated using quadratic discriminant function analysis. Sample sizes (n) are in parentheses.

Abbreviation: CI, confidence interval.

TABLE 5 Plasma testosterone (T) and estradiol-17β (E2) concentrations (ng/ml) in wild white sturgeon in the lower Columbia River, Canada

Sex	Stage of maturity	T (ng/ml)	E2 (ng/ml)
Females	Pre-vitellogenic (n = 37)	2.27 ± 0.50^{a} (0.12–12.55)	0.10 ± 0.02^{a} (0.05–0.80)
	Early to mid vitellogenic (n = 7)	23.77 ± 12.48^{b} (3.24–97.47)	2.54 ± 0.88^{b} (0.16–5.67)
	Late vitellogenic (n = 4)	127.32 ± 25.15^{c} (77.45–195.84)	8.93 ± 1.50^{b} (7.00–13.32)
	Atretic (n = 1)	0.37	0.05
Males	Pre-meiotic (n = 20)	1.91 ± 0.97^{a} (0.12–19.60)	0.07 ± 0.004^{a} (0.05–0.09)
	Meiotic (n = 2)	63.68 ± 7.14^{b} (56.54–70.81)	0.09 ± 0.005^{a} (0.08–0.09)
	Mature (n = 3)	79.45 ± 47.08^{b} (29.15–173.53)	0.11 ± 0.03^{a} (0.080–0.16)

Note: Data are mean (±SEM), range (min. and max.), and sample size (n) in parentheses. Different letters denote statistical differences among stages of maturity within females or males for T or E2. The single atretic wild female was not included in statistical analyses. Plasma sex steroid concentrations below the MQC of the radioimmunoassay were assigned the MQC (0.16–0.38 ng/ml for T and 0.10–0.21 ng/ml for E2). Non-detectable plasma sex steroid concentrations were assigned half of the MQC (0.08–0.19 ng/ml for T and 0.05–0.11 ng/ml for E2).

Abbreviations: MQC, minimum quantifiable concentration; SEM, standard error of the mean.

4.3 | Ultrasonography

The overall accuracy in using ultrasonography to assign sex was 57% (49%–65%) in the hatchery-origin (n = 149) and 70% (56%–81%) in the wild fish (n = 56). In hatchery-origin and wild fish, accuracy in assigning sex was higher in females compared to males (Table 4). No clear morphological or echogenic differences in gonadal tissue were found using ultrasonography, with the exception of wild females with vitellogenic ovarian follicles (Stages 3 and 4; Figure 3).

4.4 | Endoscopy

The accuracy in using endoscopy to assign sex was 98% (95%–100%) in the hatchery-origin (n = 131) and 100% in the wild fish (n = 7; Table 4). Ovaries with small translucent oocytes and ovigerous folds were apparent in non-reproductive females (Figure 4), and larger, vitellogenic ovarian follicles were apparent in wild reproductive females. Testicular tissue appeared smooth, white in color, and often vascularized (Figure 4).

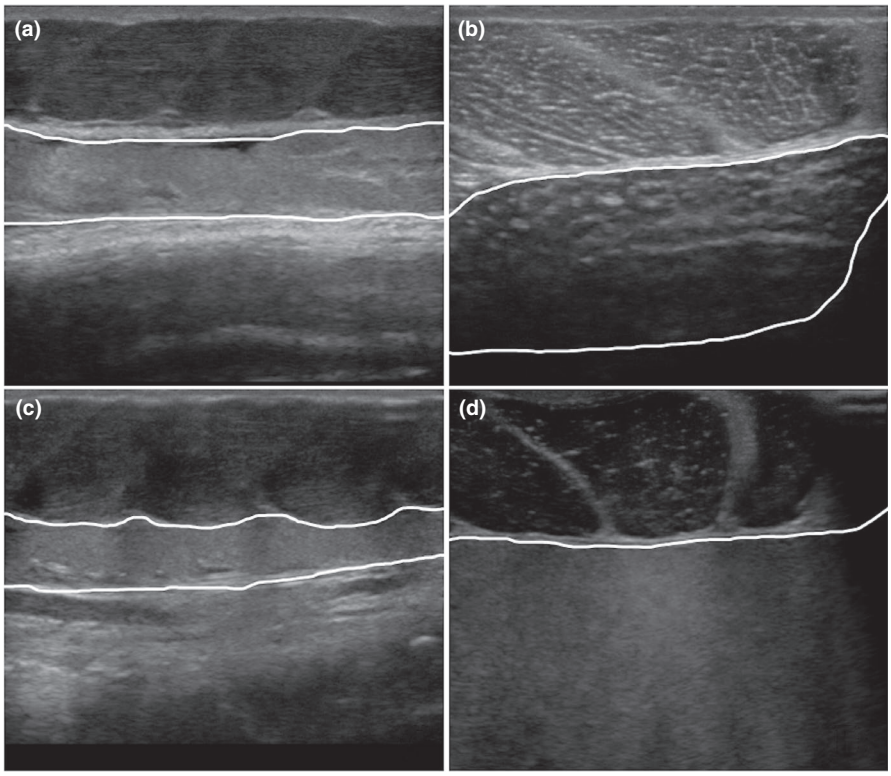

FIGURE 3 Sonograms of a (a) pre-vitellogenic (Stage 2) hatchery-origin female, (b) late vitellogenic (Stage 4) wild female, (c) pre-meiotic (Stage 2) hatchery-origin male, and (d) meiotic (Stage 4) wild male white sturgeon in the lower Columbia River, Canada. The white lines delineate gonadal tissue. The testicular tissue extends beneath the white line in (d)

4.5 | Gonadal biopsy

Within hatchery-origin fish, 335 of the 347 tissue samples contained germ cells resulting in a gonadal biopsy accuracy of 97% (94%–98%). Of the 80 tissue biopsies collected in wild fish, only 75 biopsies contained germ cells resulting in 94% accuracy (86%–98%). The biopsies without germ cells were collected from both females and males based on visual assignment of sex using endoscopy and contained somatic cells including adipose tissue. Fish without germ cells in the gonadal biopsy were not used for further analyses.

4.6 | Population reproductive structure as assigned by histology

The population reproductive structure was described in 332 hatchery-origin fish with germ cells. A total of 174 female and 158 male hatchery-origin fish were identified in 2017 and 2018. Three fish were classified as intersex with both female and male germ cells, and 12 fish were unknown as no germ cells were identified in the biopsy. All hatchery-origin fish were pre-vitellogenic females (Stages 1 and 2; Table 1; Figure 5) or pre-meiotic males (Stage 2; Table 1; Figure 5) and have yet to reach puberty.

The sex and stage of maturity was assessed in a total of 75 wild fish. Of the 49 wild females, 37 were pre-vitellogenic (Stage 2), eight were early to mid-vitellogenic (Stage 3), four were late vitellogenic (Stage 4), and one was undergoing follicular atresia. Of the 25 wild males, 20 were pre-meiotic (Stage 2), two were meiotic (Stage 4), and three were mature (Stage 5).

4.7 | Gonadal homogeneity

Of the 36 hatchery-origin fish assessed, 33% (19%–51%) were found to have non-homogeneous gonads. All females assessed ($n = 4$) had homogenous gonads. Non-homogeneous testicular development was found in 38% (21%–56%) of males ($n = 12$ of 32; Figure 6). All males from the LCR had homogeneous testicular development. Precocious cysts were found in 12 out of the 27 hatchery-origin males collected from Lake Roosevelt, WA. Of the 12 males with gonads that had non-homogeneous testicular development, 85% (78%–94%) of the biopsies ($n = 33$ of 39) assessed were found to contain precocious cysts. The precocious cysts were predominately found at or close to the periphery of the gonadal tissue (i.e., outer edges).

5 | DISCUSSION

Hatchery-origin white sturgeon sampled in this study have yet to reach puberty in the LCR, Canada. However, the study was successful in assigning sex to hatchery-origin white sturgeon aged 10–17 years using multiple tools. In the wild, white sturgeon have been documented to become spawning capable at the ages of 12–25 years in males and 15–35 years in females (Billard & Lecointre, 2001; Hildebrand et al., 2016) and sizes of 100–150 cm fork length in males and 120–180 cm fork length in females (Billard & Lecointre, 2001; DeVore et al., 1995; Haxton et al., 2016; Welch & Beamesderfer, 1993). Hatchery-origin fish assessed in this study were of similar ages to pubertal fish in other reaches of the Columbia River but

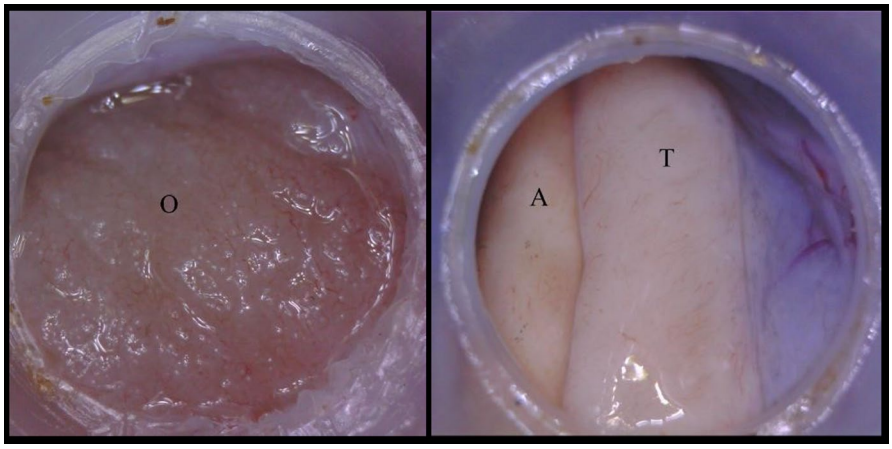

FIGURE 4 Endoscopic image of primary oocytes (O) and ovigerous folds in a prepubertal hatchery-origin female white sturgeon (left) and vascularized testicular tissue (T) and adipose tissue (A) in a prepubertal hatchery-origin male white sturgeon (right) in the lower Columbia River, Canada

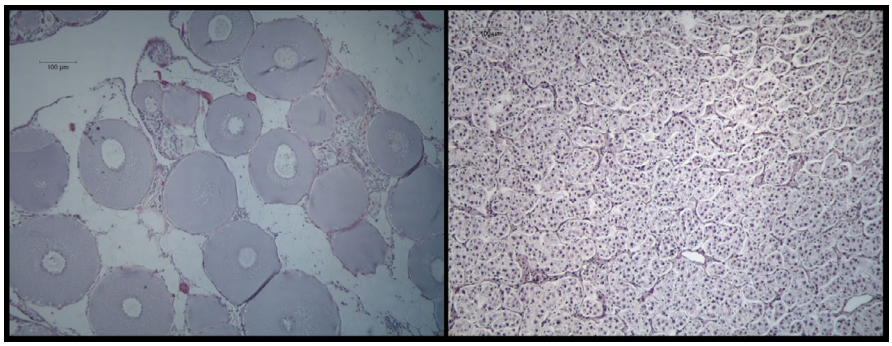

FIGURE 5 Microscopic images (10×) of ovarian tissue in a Stage 2 prepubertal hatchery-origin female white sturgeon (left) and testicular tissue in a Stage 2 prepubertal hatchery-origin male white sturgeon (right) white sturgeon in the lower Columbia River, Canada

were smaller in comparison. While sample sizes in this study were high (n = 332) compared to other work conducted on cultured and wild sturgeons (e.g., Divers et al., 2009; Masoudifard et al., 2011; Matsche et al., 2011; Munhofen et al., 2014), the fish in this study represent only a small fraction of hatchery-origin fish at large in the Transboundary Reach of the Columbia River (Crossman & Korman, 2021), underlying the importance of an annual standardized sampling program. While a balanced sampling design incorporating a range of ages was required given uncertainties in the reproductive structure of the population, we cannot conclude that there are not individuals within the hatchery-origin population that have initiated puberty as the proportion of large, older individuals assessed was limited.

We describe the efficacy of using several tools for assigning sex in prepubertal white sturgeon, emphasizing the tools with the highest accuracy rates. However, the tool chosen to assign sex and stage of maturity will depend on the objective of the study and the acceptable rate of error associated with the response being evaluated. Migratory behavior, habitat use, and sampling gear bias have been found to differ between sexes and among different classes of sex and stage of maturity in some sturgeon species (McKinley et al., 1998; Richards et al., 2014; Shaw et al., 2012, 2013), therefore assigning sex and stage of maturity with high accuracy is essential. Alternatively, programs estimating the proportion of fish capable of spawning in a given year may allow for some level of error. We demonstrated lower accuracy for the less invasive tools (i.e., ultrasonography) and attribute this reduced performance to the fact that all fish were prepubertal as higher accuracy was demonstrated with the

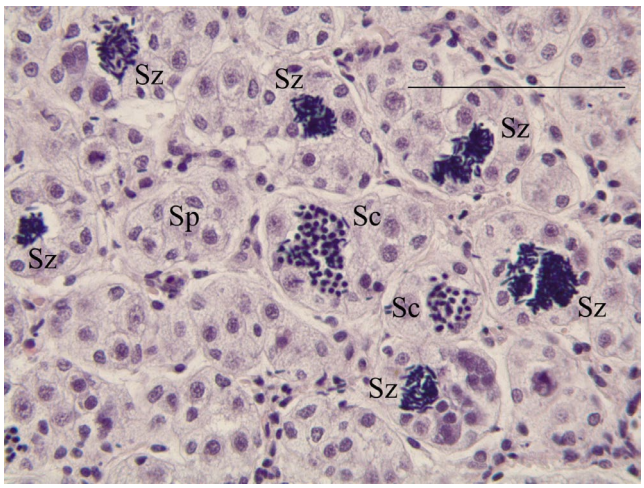

FIGURE 6 Microscopic image (40×) of non-homogeneous testicular development with precocious cysts in a Stage 2 prepubertal hatchery-origin male white sturgeon in Lake Roosevelt, Washington. The majority of testicular cysts contain spermatogonia (Sp; Stage 2; Table 1). Spermatocytes (Sc; Stage 3; Table 1) are the next stage of development following natural progression of the gonad, yet precocious cysts containing spermatozoa (Sz; Stage 5; Table 1) are present. Scale bar represents 100 μm

larger, wild fish in this study as well as in other populations that were reproductively active (e.g., Moghim et al., 2002; Webb et al., 2019; Wildhaber et al., 2007; this study). The accuracy of less invasive tools evaluated in this study, such as ultrasonography and plasma sex steroids, could continue to be reassessed in hatchery-origin populations

that are approaching puberty and cycling through gametogenesis. The recent discovery of a sex-specific genetic sequence may provide a highly accurate tool for identification of sex in sturgeons (Kuhl et al., 2020) and does not require individuals to have reached puberty, however, the tool is early in the development phase and analysis is not immediate.

Histological analysis of gonadal tissue is considered one of the most accurate methods to determine sex and stage of maturity in fishes (Blazer, 2002), including sturgeons (e.g., Chen et al., 2006; McGuire et al., 2019; Van Eenennaam & Doroshov, 1998; Webb & Erickson, 2007; Webb et al., 2002). Results from this study were similar to other research on sturgeons where histology was used to verify sex and stage of maturity (e.g., Divers et al., 2009; Matsche et al., 2011; Munhofen et al., 2014). Accuracy in using histological analysis of the gonadal tissue to assign sex has varied from 86% in pallid sturgeon *Scaphirhynchus albus* and shovelnose sturgeon *S. platorynchus* (Divers et al., 2009) to 91% in juvenile Siberian sturgeon *A. baerii* (Munhofen et al., 2014). In both those studies, accuracy was influenced by a portion of samples that did not contain germ cells, stressing the importance of recognizing gonadal tissue during sample collection which can be challenging in the field without adequate training. Lastly, while a biopsy collected in the field may be successful in collecting gonadal tissue, the tissue can also contain adipocytes (Treanor et al., 2018) and somatic cells and may not always contain germ cells, thus influencing the accuracy when assigning sex or stage of maturity.

Three intersex fish (0.9%) were identified in the hatchery-origin population. Similar to other studies with intersex sturgeon, the gonad was primarily testicular tissue with few oocytes embedded (Chapman et al., 1996; Colombo et al., 2007; Rzepkowska et al., 2014; Van Eenennaam & Doroshov, 1998). Intersex fish are common at low frequencies in both wild and cultured populations of sturgeons (Matsche et al., 2013; Rzepkowska et al., 2014; Van Eenennaam & Doroshov, 1998). The low prevalence of intersex fish in this study falls within the normal range found within other sturgeon populations, such as the white sturgeon in the San Francisco Bay (<0.01%; Chapman et al., 1996), Atlantic sturgeon *A. oxyrhynchus oxyrhynchus* in the Hudson River (1%; Van Eenennaam & Doroshov, 1998), and shovelnose sturgeon in the Mississippi River (3%; Carlson et al., 1985 and 2%; Colombo et al., 2007).

Though sturgeons are known to be gymnovarian and have synchronous gonadal development (Webb et al., 2019), the size of the gonadal biopsy (5 mm^3) required to minimize harm to live individuals is very small relative to the overall size of the gonad, which can be up to 20% of the body weight in white sturgeon (Hildebrand et al., 2016). Development of germ cells within the gonad was found to be homogenous in the small number of prepubertal white sturgeon females included in our study; however, development of germ cells in the gonads of males was non-homogenous, with some males just initiating puberty as seen by the presence of precocious cysts. Despite the presence of precocious cysts, the males sampled in this study were considered functionally immature (prepubertal) as they did not have enough spermatozoa in the testes to successfully fertilize eggs. However, they would be considered physiologically mature (post-pubertal) based on the literature (see Bennetts et al., 2019; Lowerre-Barbiere et al., 2016). To our knowledge, precocious cysts within the gonad in a highly advanced stage of gonadal development have not been described in the literature for any sturgeon or teleost species. The identification of precocious cysts, while physiologically interesting for identifying early onset of puberty, will not change the assignment of sex when the stage of maturity is assigned based on the predominant stage of gonadal development for an individual as was done in this study.

Endoscopy, although invasive, was the most accurate in assigning sex in both hatchery-origin and wild white sturgeon in this study with overall accuracy rates of 98% and 100%, respectively. These results were consistent with other studies that used endoscopy to assign sex in sturgeons (sex confirmed by histological analysis of gonadal tissue) with accuracy varying from 92% in shovelnose sturgeon (Wildhaber et al., 2005), 97% in Siberian sturgeon (Munhofen et al., 2014), and 100% in cultured Russian sturgeon *A. gueldenstaedtii* (Hurvitz et al., 2007). In order to successfully use endoscopy as a tool to assign sex or collect a gonadal biopsy, an understanding of the anatomical and morphological features of gonadal tissue in sturgeons is critical.

The pattern of T and E2 production in sturgeons allows for discrimination of sex and stage of maturity less invasively (see Webb & Doroshov, 2011), however, sex steroid concentrations remain low prior to the initiation of puberty and during the non-reproductive phase of gametogenesis once puberty has been reached (Du et al., 2017; Webb & Doroshov, 2011; Webb et al., 2002). When sturgeon have reached puberty and are in the advanced stages of gametogenesis, using plasma steroid concentration to assign sex and stage of maturity has higher accuracy (e.g., Malekzadeh Viayeh et al., 2006; Webb & Doroshov, 2011; Webb et al., 2002; this study). Based on estimates from other studies, the mean T concentrations in prepubertal females in this study appear to be similar to other white sturgeon (Feist et al., 2004; Webb et al., 2002) and Chinese sturgeon *A. sinensis* (Du et al., 2017), lower than the mean concentrations detected in similar stage females of the hybrid bester *Husu huso* × *Acipenser ruthenus* (Amiri et al., 1996), and higher than Persian sturgeon *A. persicus* (Malekzadeh Viayeh et al., 2006). The mean T concentrations in prepubertal males in this study were estimated to be lower than the concentrations detected in similar stage males of the hybrid bester (Amiri et al., 1996), Chinese sturgeon (Du et al., 2017) and white sturgeon in the LCR (Webb et al., 2002) but similar to concentrations estimated in shovelnose sturgeon (Stahl et al., 2009). Notably, the mean concentrations of T among the females and males in these species were not biologically relevant. Further, comparisons of steroid concentrations among sturgeons is challenging as specific concentrations are often not reported in the literature. Rather, relationships are provided graphically which requires an estimation of the true concentration and specific concentrations are warranted in future studies.

Ultrasonography has been growing in popularity due to advancement and accessibility of the technology and the non-invasive nature

of the tool (Chebanov & Galich, 2009; Webb et al., 2019). Within this study, ultrasonography had the lowest accuracy of all tools tested. Potential explanations for lower accuracy may be the limited number of older, larger fish assessed and the limited training time prior to application of the tool. The smaller ages and sizes of the hatchery-origin fish incorporated into the study suggests gonadal tissue may be less developed, small in size, or both, influencing the readers ability to distinguish between tissue types. Other studies have also found it difficult to differentiate between less developed gonadal tissue, which lacks distinguishing morphological features (sex confirmed at necropsy; Moghim et al., 2002; sex confirmed using histological analysis of gonadal tissue; Munhofen et al., 2014; Wildhaber et al., 2007). Despite challenges in the field with prepubertal fish, some studies have been successful in using ultrasonography to assign sex to prepubertal cultured sturgeon populations (97% female and 98% male three-year-old beluga sturgeon *H. huso*, Masoudifard et al., 2011; 89% in three-year old and 84% in four-year old Siberian sturgeon, Munhofen et al., 2014). Of note, cultured sturgeon programs typically have fish growing at rates that accelerate development at younger ages, and managers are applying ultrasonography to significantly higher numbers of fish (e.g., >1000/day; Chebanov & Galich, 2009, 2018) compared to this study allowing for more training opportunities. Higher accuracy rates have been reported when using ultrasonography to assign sex to sturgeons with developing gonads due to identifiable features such as decreased adipose tissue and vitellogenic ovarian follicles in female sturgeons (Chiotti & Boase, 2016; Colombo et al., 2004; Du et al., 2017; Moghim et al., 2002). Within this study, overall accuracy was higher in the post-pubertal, wild fish compared to the hatchery-origin fish. While there is promise for ultrasonography, assigning sex in small (<1.0 m) prepubertal white sturgeon will remain challenging in the field without extensive training and advancement of technology (e.g., higher resolution transducer).

The hatchery-origin white sturgeon assessed in this study had not yet reached puberty as of 2018, though similar aged fish in the United States portion of the population have (M. Webb, unpublished data). Standardized annual monitoring will be important to describe how the reproductive structure of hatchery-origin populations changes over time (e.g., age and size at puberty, sex ratio, condition) and to determine when the fish start to introgress with wild spawners. Population reproductive structure is important for management and can help partition variability in growth or condition attributed to energy allocated to reproduction.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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