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Histological characterization of gonadal development of juvenile Lake sturgeon (*Acipenser fulvescens*)

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Abstract Lake Sturgeon (*Acipenser fulvescens*) hatcheries that produce juveniles to augment natural populations are of increasing importance to management and species recovery. Maintaining balanced sex ratios of juveniles that are released into the wild is important to future natural reproduction. Greater understanding of the chronology of gonadal development of hatchery-reared fish can help managers target ages to conduct surveys, predict cohort sex ratios, and maintain demographically healthy adult populations. We histologically characterized gonadal development of juvenile Lake Sturgeon between the ages of three to 53 months. Gonads of all surveyed fish ($N = 56$) were undifferentiated and immature at all time-points surveyed until 41 months, although histological changes consistent with gonadal enlargement and development were observed (e.g., increase of adipose tissue, gonadal epithelial ridge thickening). At 41 months, sex could be determined in two (25%) of the eight sampled fish based on presence of ova within the gonadal tissue, and five (62.5%) showed continued gonadal development, but were still undifferentiated (sex could not be assigned). At 53 months, sex could be determined in six (75%) of eight sampled fish. Two (25%) showed advanced development (e.g., epithelial thickening of the germinal ridge), but the gonads were undifferentiated (i.e., no ova or spermatozoa present). Delayed gonadal differentiation is consistent with the delayed sexual maturity generally observed in Lake Sturgeon relative to other teleost species. Sample sizes precluded statistical evaluation, however, sex determination did not appear to be associated with egg incubation temperature.

Keywords Lake sturgeon · *Acipenser fulvescens* · Sex determination · Histology · Gonad maturation

Introduction

The developmental chronology of gonadal differentiation is a fundamental aspect of biology in which undifferentiated gonadal tissues transition to testes or ovaries. Among fishes, the timing of gonadal differentiation is highly variable, with some species showing differentiation at or near hatch (e.g., *Cynopoecilus melanotaenia*; Arezo et al. 2007), within a few weeks (e.g., 27 days in Coho salmon *Oncorhynchus kisutch*; Piferrer and Donaldson 1989), while for other species more than a year is required for gonadal differentiation to be

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detectable using microscopy techniques (e.g., 20 months in Adriatic Sturgeon *Acipenser naccarii*; Grandi and Chicca 2008). Importantly, the development of organisms is influenced by both biotic (e.g., conspecific density and operational sex ratio) and abiotic factors (e.g., temperature and food availability) that can affect not only the outcomes, but also the timing of development. Greater understanding of the chronology of gonadal development is of particular interest for hatchery-reared fish, as information pertaining to impacts that rearing conditions have on gonadal development can help managers target ages to conduct surveys, predict cohort sex ratios, and maintain demographically healthy adult populations.

Conservation and management of fish populations often relies on stocking to produce fish that will survive and reproduce in the wild (Trushenski et al. 2014). Of central importance to this goal is the production of viable offspring that survive into adulthood. A major focus of current research serves to identify optimal rearing conditions that maximize growth and survival while maintaining hatchery production efficiency (Bauman et al. 2015). For example, abiotic parameters (i.e., rearing temperature) are often controlled to create the environmental conditions that synchronize hatching times (Laurel et al. 2008). However, controlling environmental conditions can impact gonadal development, and in species with environmental sex determination (ESD), managers run the risk of producing offspring with biased sex ratios, which could have negative effects on future population recruitment and viability (Larsson and Förlin 2002), effective population size, and levels of genetic diversity (Frankham 1995).

Environmental sex determination (ESD) is widespread among fishes (Craig et al. 1996; Valenzuela and Lance 2004; Ospina-Álvarez and Piferrer 2008; Baroiller et al. 2009), and can result from differences in temperature experienced during thermal-sensitive periods (TSPs; Harrington 1967; Conover and Kynard 1984; Baroiller and D'Cotta 2001), alterations in pH during development (Rubin 1985; Baroiller and D'Cotta 2001), or through social conditions (including density and skewed sex ratios) experienced by juveniles or adults (Davey and Jellyman 2005). For species with ESD, homogenization of environmental conditions can lead to production of populations with skewed sex ratios that can compromise goals for long-term population sustainability. Concerns are particularly notable for long-lived species with delayed sexual maturity, because years may pass following release of hatchery fish before sex ratios of returning adults are known. For species of conservation concern with ESD, or where sex determination mechanisms are unknown or poorly understood, the threat of skews in sex ratios stemming from temperature changes associated with climate (Kamel and Mrosovsky 2006; Hawkes et al. 2007; Ospina-Álvarez and Piferrer 2008) or artificial propagation such as crosses and rearing within a hatchery setting (Crossman et al. 2011) are widely recognized.

Lake Sturgeon (*Acipenser fulvescens*) is a long-lived, iteroparous spawning fish characterized by delayed sexual maturity. The sex-determining mechanism of Lake Sturgeon is unknown. Evidence for genetic sex determination has not been documented despite the utilization of multiple genetic and molecular approaches (Wuertz et al. 2006; Keyvanshokooh et al. 2007; McCormick et al. 2008). To date, no specific sex chromosomes are known in sturgeon, no sex-specific genes have been identified, and although sex-based differences in gene expression were found in two potential candidate genes (*DMRT1* and *TRA-1*; Hale et al. 2010), factors influencing differential gene expression leading to sex have not been evaluated.

Lake Sturgeon have experienced significant declines in abundance and distribution over the past 150 years (Holey et al. 2000). Currently less than 1% of historical populations remain in the Great Lakes region (Hay-Chmielewski and Whelan 1997). As a result, management and conservation of Lake Sturgeon has focused on hatchery production to supplement existing or repatriate extirpated populations (Holey et al. 2000; Welsh et al. 2010). Historically, traditional hatchery programs for Lake Sturgeon reared fish in water of constant temperature (ground water) during early developmental periods. Lake Sturgeon populations supplemented by traditional hatchery facilities during this period are characterized by skewed sex ratios. For example, from 1983 to 1988 Black Lake (Cheboygan County, MI) was stocked with 11,472 Lake Sturgeon that were raised in a constant temperature ground water (11 °C) from egg fertilization to hatch, 15 °C until they began feeding, and 20 °C thereafter until release. When these stocked fish reached sexual maturity and began spawning in the Black River in the early 2000's, most of the presumed first-time spawners were males ($\leq$120 cm TL) and very few were females ($\leq$142 cm TL) (Michigan Department of Natural Resources and Michigan State University, unpubl. data).

The earliest timing that sex can be determined in Lake Sturgeon is unknown, and no external morphological characteristics are associated with sex in developing juveniles. The objectives of this paper are to characterize the histological changes in gonadal development and maturation from hatchery-produced Lake Sturgeon from three months through 53 months of age to determine the earliest time when sex could be determined. The chronology of appearance of histological features characterizing males and females through early ontogenetic periods are identified. Because the most common form of ESD in vertebrates is Temperature Sex Determination (TSD) we used different egg rearing temperatures that allowed for preliminary evaluation of the effects of rearing temperature on juvenile Lake Sturgeon gonadal development and sex differentiation.

Methods

Field collection

During the 2012 spawning season, adult Lake Sturgeon were captured in the upper Black River (UBR) in Cheboygan County, Michigan using long-handled dip nets. Gametes were collected from four females and four males on May 3rd and 4th by applying pressure on the lateral abdominal areas and extruding sperm and eggs through the urogenital opening. Eggs were placed in plastic bags and stored in ambient temperature river water. Sperm was collected using 5-mL syringes and placed on ice in the field. Gametes were transported to a streamside rearing facility and fertilizations were conducted within twelve hours of collection. Eggs (~200 per female) were fertilized with 0.5 mL of sperm from a single male to produce 4 full-sibling families. Containers made from PVC and fine-mesh screen (31.90 cm^2) were used to keep eggs from different families separate during incubation.

Temperature treatments

Fertilized eggs were acclimated to treatment temperatures by adjusting temperatures two-degrees Celsius per hour before being placed into temperature-controlled heath trays. Fertilized eggs were incubated in heath tray stacks where temperature was controlled to produce two constant temperatures [cold (10 $\underline{+}$ 1 °C) and warm (~18 $\underline{+}$ 1 °C), mean $\underline{+}$ STD], representing temperatures characterizing the early and late spawning periods, respectively in the UBR (Forsythe et al. 2012). We used an Aqua Logic Trimline Delta Star 1/4HP Chiller TLD-3 with Temperature Controller, and a Process Tech Heater, Single-Phase, "L" Style 1800 W, 115 V, 15 amps, 16" x 24" Process technologies DRAE15–1 EASYPLUG LCD digital temperature control, both with ± 1 °C accuracy. Water temperature was measured daily using Onset HOBO pressure loggers (Cape Cod, Massachusetts, USA).

Rearing from hatch to juvenile period

Following hatch, individuals from each family were kept separately during the first three months. From hatch to approximately 40 days post-hatch, larvae from all females and both temperature treatments were housed separately by family in 3 L aquaria at the Black River Streamside Rearing (BRSR) facility in Cheboygan County, Michigan at ambient river temperatures. From the onset of exogenous feeding to approximately four weeks post-exogenous feeding larvae were fed *Artemia nauplii*. After four weeks, individuals were transferred to 3 ft. circular tanks and were fed a diet of chironomidae larvae through the remainder of time until the first group of individuals were euthanized. During April through August juveniles were raised at the Black River Streamside Facility. Water was pumped directly from the UBR and thus temperatures varied in accordance with ambient stream temperatures (between 10 and 26 °C). During August of the first year (2012), individuals were tagged with 12.5 mm 134.2 kHz full duplex Biomark ISO FDX_B passive integrated transponder (PIT) tags so all individuals could be uniquely identified. After PIT tag insertion, all individuals from both warm and cold incubation temperature groups were combined into a common rearing tank. In September, fish were transported to the Michigan Department of Natural Resources Wolf Lake Hatchery in Allegan County, Michigan and were reared there from mid-September through early April each year. The Wolf Lake Hatchery used ground water at a constant year-round temperature of 10 °C.

Histological collection

Juveniles ($N = 56$) were euthanized using a lethal dose of tricaine methanesulfonate (MS-222; 500 mg/L) according to approved Michigan State University Animal

Use and Care guidelines. A total of eight fish were euthanized for each age group (3 m, 6 m, 12 m, 17 m, 29 m, 41 m and 53 m; N = 56 total). Of the eight fish in each group, four samples represented fish where the eggs were incubated in warm conditions (18 °C) and four fish from the cold (10 °C) egg incubation treatments. Immediately following euthanasia, gross dissections were made to isolate gonadal regions and surrounding organs and to expose tissue for rapid fixation of internal structures. Sectioning of juvenile sturgeon at three and six months of age was based on external characteristics with cuts made immediately posterior to the cloaca and between the 3rd and 4th ventral scutes (therefore the section included the cloacal opening). Juveniles sacrificed at 12, 17, and 29 months had thicker skin and the more pronounced thickening of bony plates relative to previous samples that reduced formalin penetration and required additional gross dissection (more sections were taken) to expose the internal organs. All samples younger than 41 months of age were decalcified in a 14% EDTA/dH_2O solution prior to sectioning. Juvenile samples at 41 months and older, were sufficiently large to excise the gonadal tissue directly and eliminated the need for decalcification. A ventral incision was made beginning at the vent (cloaca) to expose the gonadal tissue. Gonadal tissue with immediately surrounding organs were removed and processed. Dissected tissues were fixed in 10% formalin and decalcified at room temperature. Tissues were embedded in paraffin and sectioned at five microns using a Reichert Jung 2030 rotary microtome. Sections were mounted to slides and stained using Hematoxylin and Eosin (H&E; Luna 1968) using a Leica CV 5030 (Leica Biosystems, Buffalo, IL). Sections were examined under an Olympus AH2 light microscope using SPOT software 3.5.9 (Diagnostic Instruments, Inc., Sterling Heights, MI).

Reference samples

Each year, a small number of angler harvest permits are made available to the public in accordance with State and Tribal allocations. Gonadal tissue from known male and female sturgeon were collected from adults taken in the harvest to use as reference tissue of male and female Lake Sturgeon. Females show an abundance of primary oocytes contained within the gonadal tissue (Fig. 1, a-c), and males show tubules contained within the testes tissue (Fig. 1, d-f).

Results

A time series of histological changes of the gonadal tissue is provided below. Some sampling time periods yielded similar results for levels of development and differentiation and were subsequently combined in the text, with notable changes described within. A time series of five major sampling periods for three and six months, twelve and seventeen months, and independent characterization for twenty-nine, forty-one, and fifty-three months is provided below.

Three and six months

At three months gonads were at an undifferentiated state (Fig. 2a-c). In both cold and warm incubated fish, the gonadal ridge could be identified and was lined with simple cuboidal epithelium. At six months, gonadal examination showed an increase in gonadal tissue size relative to the three month samples (Fig. 3a, b), however the gonads were still undifferentiated (thin gonadal ridge, no prospective primordial oocytes or duct formation). Increase in gonad size was associated with an increase in adipose tissue within the gonad, but no demonstrative epithelial changes were observed.

Twelve and seventeen months

At twelve months, gonads were undifferentiated, but with notable differences relative to fish at six months of age. Gonadal epithelium changed from simple cuboidal to pseudostratified along the gonadal ridge in some, but not in all samples. Epithelial changes observed were consistent with females (simple cuboidal to pseudostratified) in another species of sturgeon (Shortnose Sturgeon *Acipenser brevirostrum*; Flynn and Benfy 2007). However, in the seventeen month fish, tubular duct structures consistent with potential male gonadal development were also identified in the presence of the pseudostratified epithelium (Fig. 4). Thus, pseudostratified epithelium is not a reliable early-female indicator for Lake Sturgeon. At seventeen months, the trend toward gonadal differentiation was not consistent throughout the gonad with more differentiation (e.g., thickening of gonadal ridge) observed in more anterior sections relative to more posterior sections within the same individual.

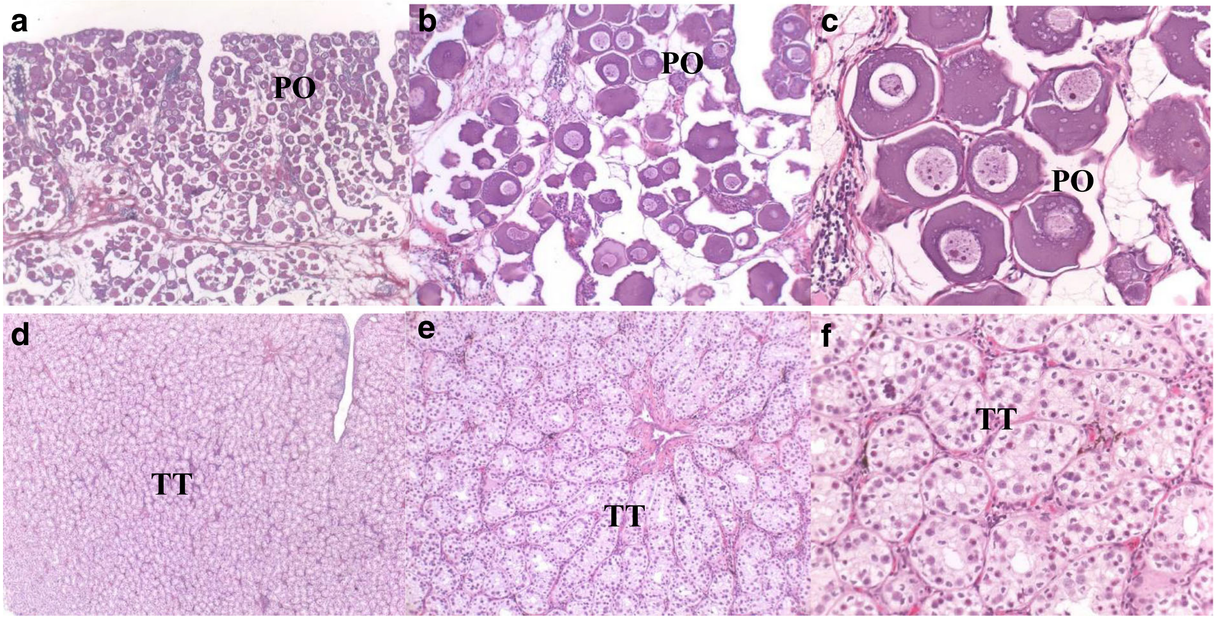


Fig. 1 Reference gonadal tissue adult females (A-C, 2.5X, 10X, and 25X respectively) showing primary oocytes (PO) and adult male (D-F, 2.5X, 10X, and 25X respectively) gonadal tissue showing tubule formation (TT) in testes tissue

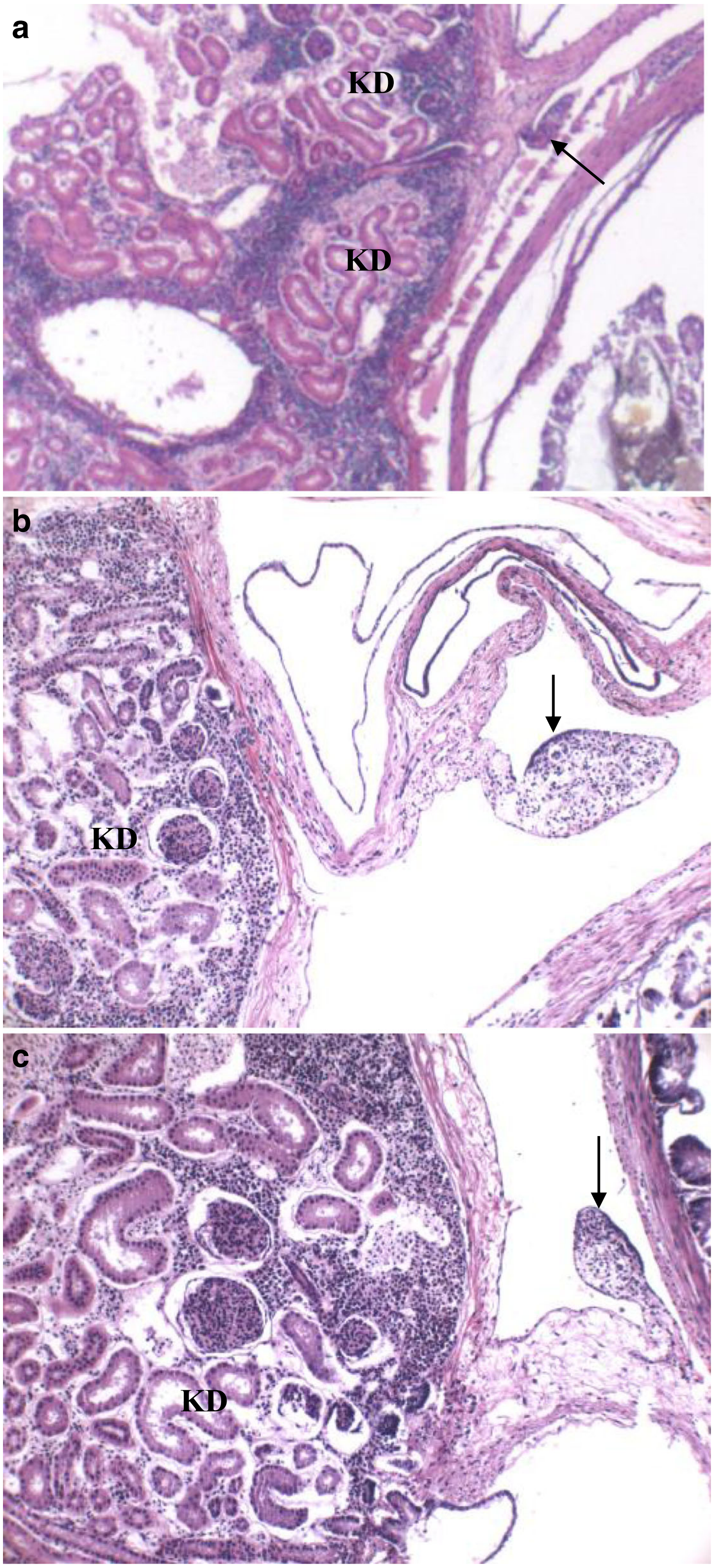
Twenty-nine months

At twenty-nine months, gonads were undifferentiated, but notable differences relative to the seventeen month samples were observed. In contrast to the seventeen month samples, where epithelial changes were only observed in a subsample of fish, at twenty-nine months evidence of gonadal epithelial differentiation was found in all samples. All samples were characterized by a large amount of adipose tissue within the gonad, along with differential epithelial changes between the fatty end and the gonadal ridge (Fig. 5). At twenty-nine months, some epithelial changes were observed in fish that were consistent with sexual development. For example, in one individual, the gonads showed extensive folding along the gonadal ridge, with pseudostratified epithelium and large proportions of both brown and white adipose tissue. Similar to samples collected at seventeen months, in a subset of the twenty-nine month old samples, changes in the gonadal epithelia from simple cuboidal to pseudostratified were observed. These changes are thought to be associated with female development (see Flynn and Benfy 2007), but in our fish the epithelial changes occurred in samples that also had duct differentiation consistent with male development (extensive ducts within the gonadal tissue). A time-series comparison of gonadal changes from 3 months through to 29 months is provided in Fig. 6, showing an increase in size, adipose tissue, and changes to the gonadal ridge with advancing time.

Forty-one months

Five of the eight (62.5%) fish sampled showed advanced development and differentiation relative to the twenty-nine month sampling, however the gonads were still undifferentiated. One fish (12.5%) that was comparatively smaller exhibited developmental features consistent with the twenty-nine month old fish. Sex could be determined in two (25%) of the eight fish sampled. The two fish in which sex could be determined were both female and demonstrated clear identifiable, unambiguous primary oocytes present in well-defined ovarian tissue (Fig. 7). In all five samples showing advanced differentiation (e.g., presence of thickened gonadal ridge, pseudostratified epithelia), variation with regard to the degree of gonadal differentiation within an individual was observed, with more differentiation occurring more anterior relative to posterior sections of gonadal tissue. In all five samples, the presence/absence of pseudostratified epithelia versus simple cuboidal epithelia within the gonadal ridge did not coincide with sex identification. Notable differences in the clustering of early-

Fig. 2 Gonadal tissue from juvenile Lake Sturgeon (3 month) under (**a**) 2.5X (gonad at arrow; KD is kidney) (**b**-**c**) 10X magnification (arrow at gonadal ridge)



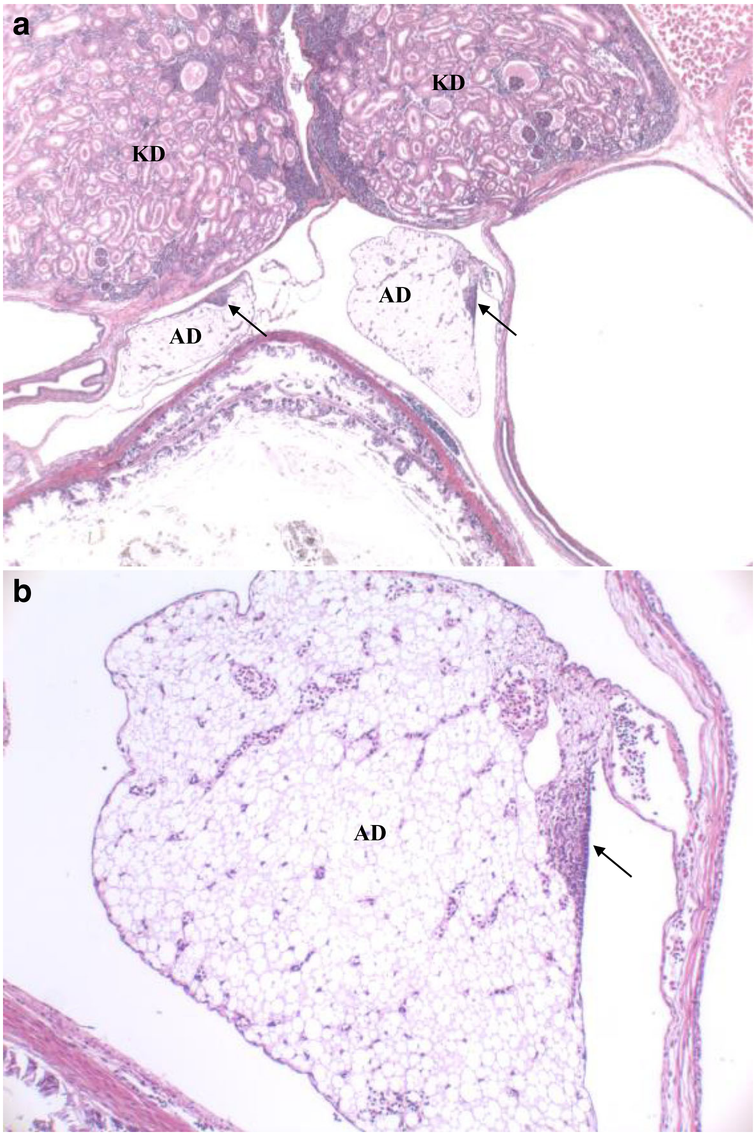


Fig. 3 Gonadal tissue from juvenile Lake Sturgeon (6 month) under **a** 2.5X and **b** 10X magnification shows an undifferentiated gonad that is larger in size and with more adipose tissue relative to the 3 month old fish (arrows indicate gonad with arrow placed at the gonadal ridge, KD is kidney, AD is adipose tissue within the gonad)

differentiation state cells within the gonadal tissue were observed. Some samples showed enlarged and distinct cells clustered into bundles within the gonadal tissue (Fig. 8b, d, f) whereas other samples showed these gonadal cells arranged into string-like patterns (Fig. 8a, c, e).

In two of the five (40%) undifferentiated fish we noted the start of compartmentalization within the gonadal region, consistent with posterior locations in the two female fish. One fish sampled had immature gonadal tissue, consistent with its body size and developmental status characteristic of twenty-nine month old fish.

Fifty-three months

At fifty-three months, gonads were differentiated in six of the eight fish (75%) sampled and could be reliably assigned a sex based on the presence of primary oocytes (female) or tubule formation consistent with testes tissue (male). Three of the sampled fish were females and showed clear evidence of ova development within the gonadal tissue (e.g., Fig. 9). Males showed delayed gonadal development relative to females. Only one male fish sampled showed evidence of early spermatagonia. Two males showed evidence of tubular and duct formation consistent with male samples (Fig. 9b), however

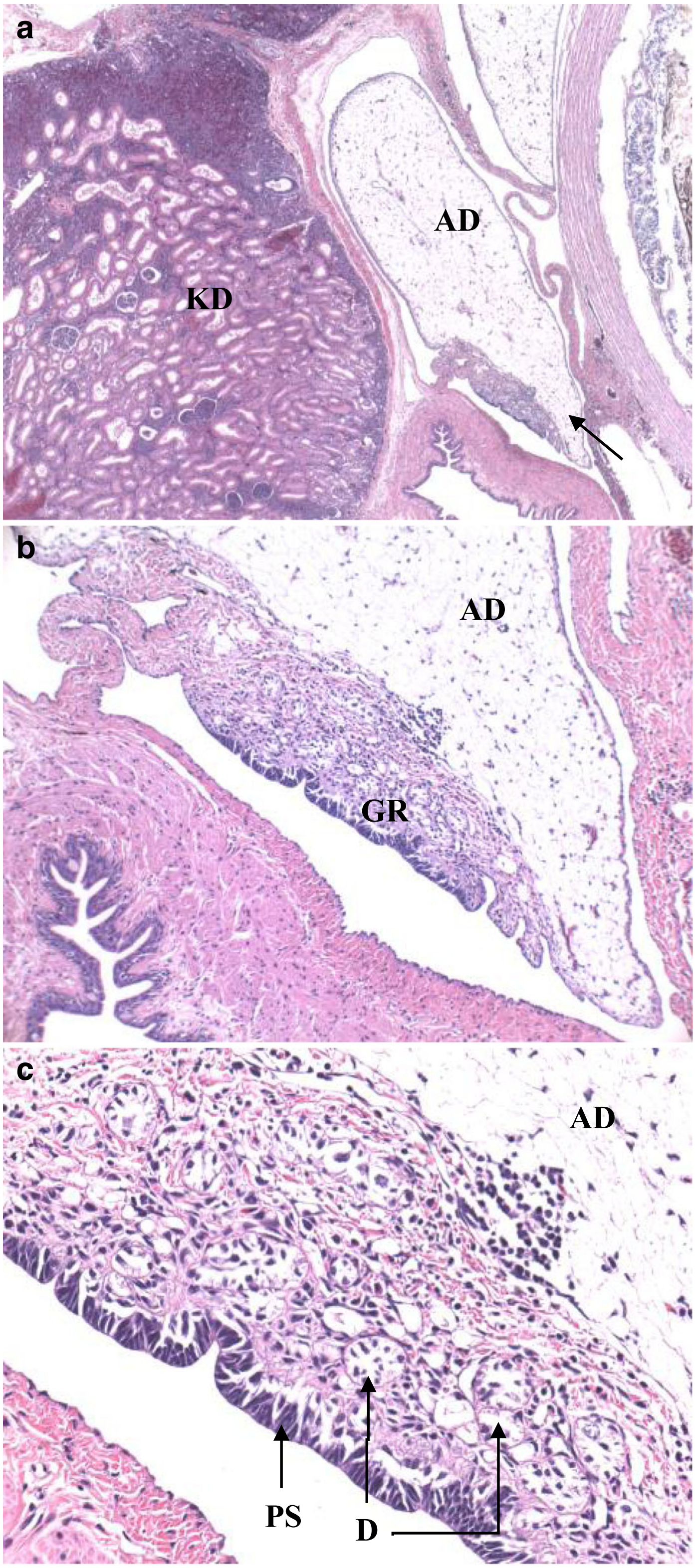


Fig. 4 Gonadal tissue from juvenile Lake Sturgeon (17 months) under **a** 2.5X, **b** 10X, and **c** 25X magnification showing an increase in size, adipose tissue and epithelial differentiation relative to the 6 month old fish (arrows indicate gonad, KD is kidney, AD is adipose tissue within the gonad, GR indicates the gonadal ridge). Note: the pseudostratified epithelia (PS) and presence of potential ductwork (D) within the gonad along the gonadal ridge

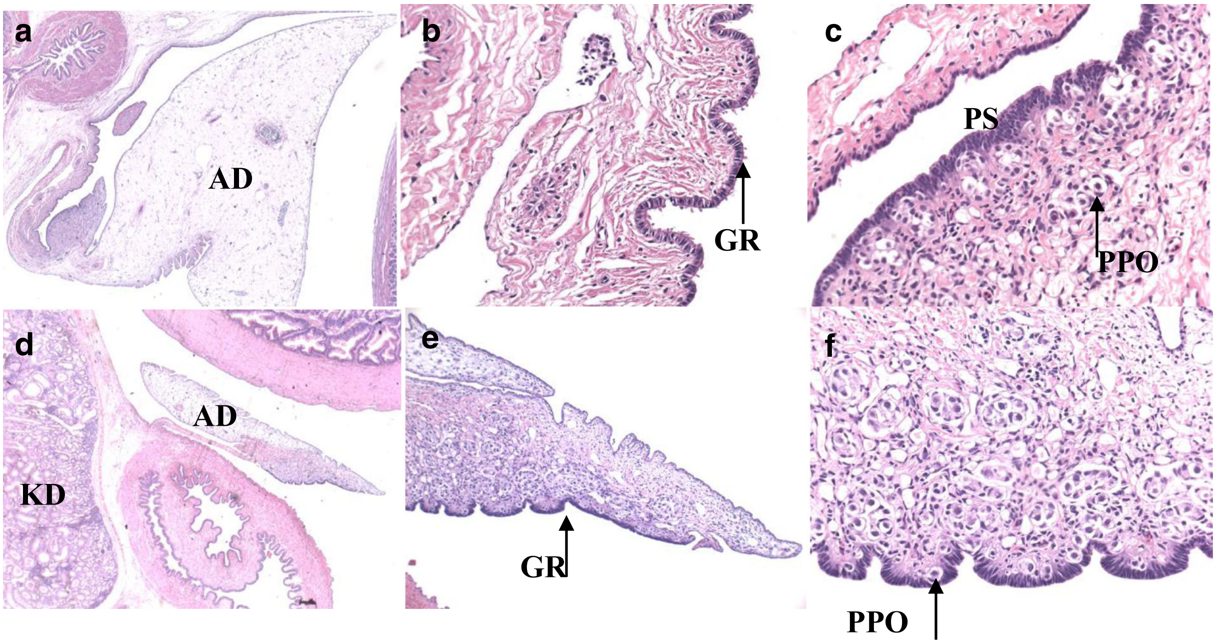


Fig. 5 Gonadal tissue from juvenile Lake Sturgeon (29 months) under **a** 2.5 **b** 10X and **c** 25X magnification showing an increase in size, adipose tissue (AD) and differentiation relative to the 17 month old fish **d** 2.5X, **e** 10X, and **f** 25X. Specifically of note are the epithelial changes and presence of potential ductwork within the gonad along the gonadal ridge or the presence of thickened pseudostratified epithelium (PS) with prospective primordial oocyte (PPO) development contained within the gonadal tissue at the gonadal ridge (GR). Kidney (KD) labeled for reference

mature spermatagonia were absent. Two of eight fish (25%) showed advanced development and differentiation (increase in size, increase in adipose tissue, and thickening of gonadal ridge) compared to twenty-nine month fish sampled, however gonads were still undifferentiated.

Evaluation of temperature effects was restricted to only 53 m fish samples ($N = 8$), due to a lack differentiated gonads in previous ages. Although sample sizes were too small to statistically evaluate differences in sex ratios as a function of incubation temperature, there did not appear to be any relationship between egg incubation temperature and sex at fifty-three months. (Table 1). Size (total length mm) at fifty-three months is not a useful indicator of gonadal development. Fish that could not be assigned a sex were similar size (mean $\pm$ stdev = 745.5 $\pm$ 19.1 mm, min-max = 732–759 mm) as females (mean $\pm$ stdev = 741.33 $\pm$ 37.31 mm, min-max = 711–783 mm) and larger than males (mean $\pm$ stdev = 703.3 + 13.6 mm, min-max = 695–719). Temperature treatment was not associated with overall body size at the time of sampling among 53 month old fish. Mean size among cold-incubated fish was 729.5 mm compared to 727 mm among warm-incubated fish (cold min-max = 696–759 mm, stdev = 25.81; warm min-max = 695–783 mm, stdev = 38.64).

Discussion

Histological characterization of sex in Lake Sturgeon is not feasible prior to 53 months post-hatch, which is delayed relative to other Acipenseriform species. In the absence of evidence of genetic sex determination, investigators have used histological examination, gene expression for sex-specific markers, and hormone assays to determine sex. Webb et al. (2002) were able to reliably assign sex of wild-caught white sturgeon *(Acipenser transmontanus)* using hormonal assays from plasma and validated using histological characterization of both mature and immature fish, but with unestablished ages. Grandi and Chicca (2008) were able to reliably determine sex of Adriatic Sturgeon (*Acipenser naccarii*) at approximately 20 months of age by histological examination of gonad tissue. Berbejillo et al. (2012) were able to determine sex of male Siberian Sturgeon (*Acipenser baerii*) by assaying cell transcription factors associated with testis

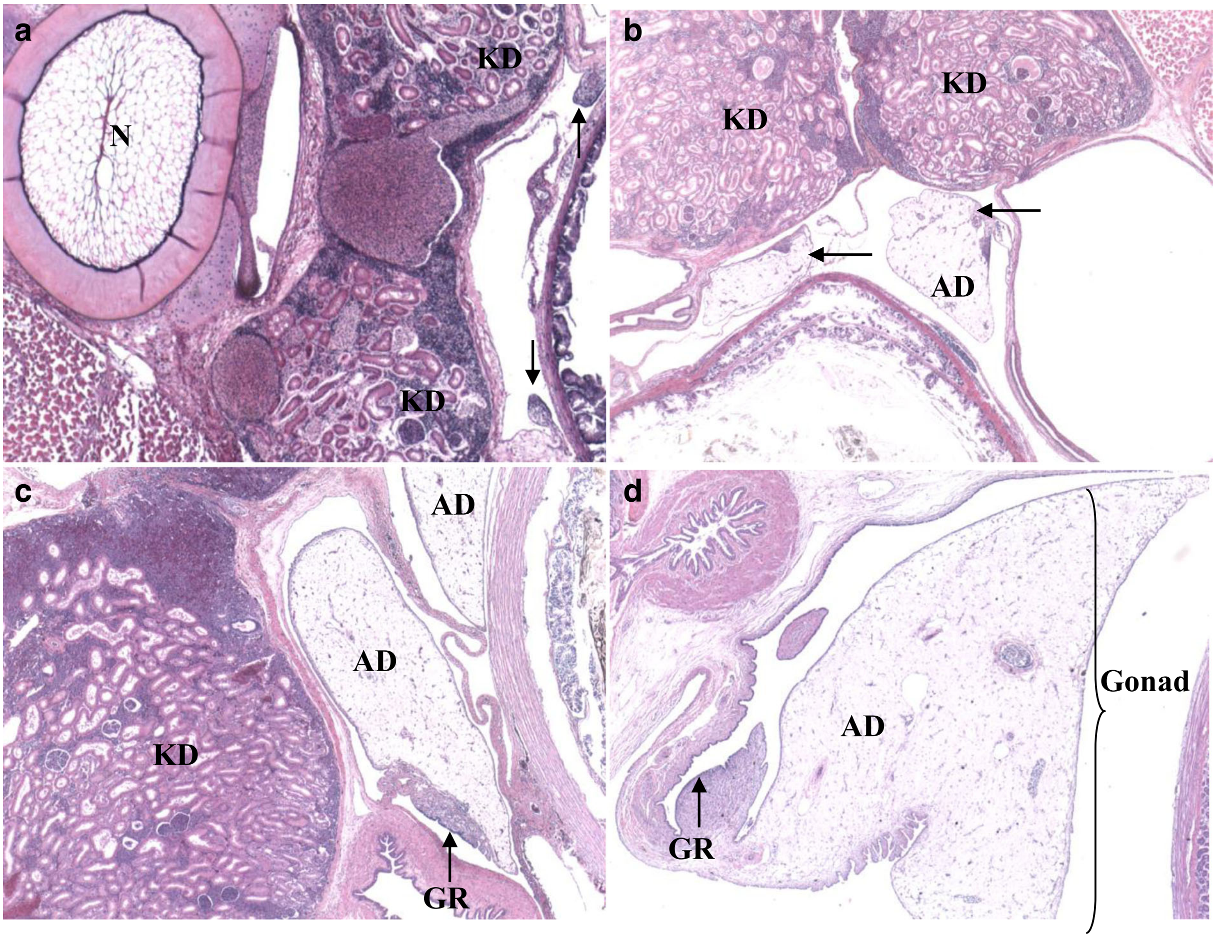


Fig. 6 Time series showing gonadal changes at **a** 3 months, **b** 6 months **c** 17 months, and **d** 29 months; all images at 2.5X magnification (N = Notochord, KD = kidney, AD = Adipose Tissue, arrows show gonad, GR is gonadal ridge)

development as early as 16 to 18 months of age. Chen et al. (2006) were able to differentiate sex of Chinese Sturgeon (*Acipenser sinensis*) by nine months of age using histological examination. Finally, Flynn and Benfy (2007) histologically identified early gonadal changes associated with sex in Shortnose Sturgeon (*Acipenser brevirostrum*) as early as 6 months of age, and could assign fish to male or female at 15 months. Our data suggest Lake Sturgeon (*Acipenser fulvescens*) sex differentiation based on histological examination is not possible until fish are 41 to 53 months old.

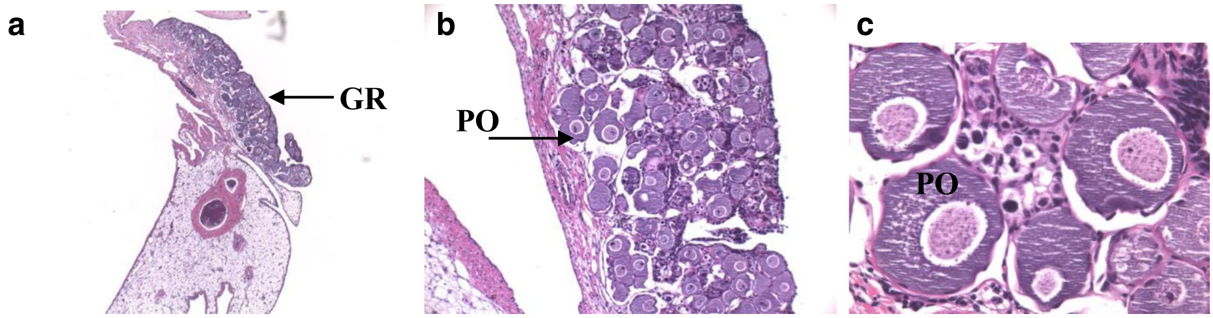


Fig. 7 A female identified histologically at 41 months under **a** 2.5X, **b** 10X, and **c** 40X magnification showing the gonadal ridge (GR) and definitive presence of primary oocytes (PO)

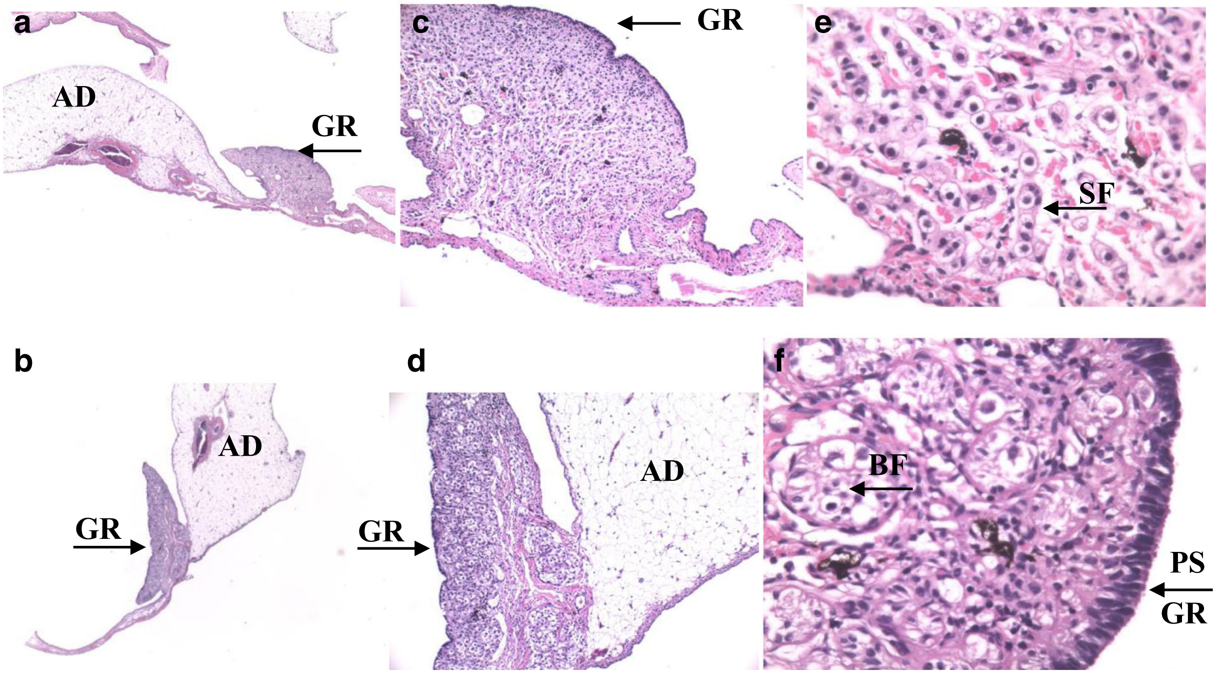


Fig. 8 Immature 41 month old fish under **a-b** 2.5X, **c-d** 10X, and **e-f)** 40 X magnification where the developing gonadal tissue shows distinction between string formation (SF; image (**e**) and bundle formation (BF; image **f**) with the gonads showing increased adipose tissue (AD), thickening of gonadal ridge (GR) and pseudostratified epithelium (PS)

Consistent with species differences in age at maturity, Lake Sturgeon gonadal development is substantially delayed relative to other sturgeon species (Table 2). Gonadal changes associated with sex were not well defined until 41 months, and the majority of individuals did not exhibit sex-specific tissue differentiation until 53 months. Lake Sturgeon gonadal development does not appear to differentiate over the same ontogenetic time scale as has been described in other sturgeon species. Further, early indicators of sexual development appear to be inconsistent across species. For example, although the presence of pseudostratified epithelia in the gonadal ridge was associated with female gonadal development in Shortnose Sturgeon (Flynn and Benfy 2007), we observed pseudostratified epithelia in the presence of tubual development associated with testes development in Lake Sturgeon. While an alternative could be that the individual was hermaphroditic (as observed in Shovelnose Sturgeon, *Scaphirhynchus platorynchus*; Colombo et al. 2007), the results highlight that caution should be used when extrapolating results among species using secondary sexual development and characteristics (including histological changes) that were established for a single species.

The variation in timing of gonad development among sturgeon species is not unexpected given the substantial variation in timing of sexual maturation among sturgeon species. Lake Sturgeon fall towards the upper end of the spectrum, with males maturing at 12 to15 years and females maturing at 18 to 27 years (Table 2; Harkness and Dymond 1961; Scott and Crossman 1973; Bruch et al. 2001). In contrast, male Adriatic Sturgeon mature at age 6 to 8 years. (females were still immature at age 10 years; Grandi and Chicca 2008); male and female Siberian Sturgeon both mature as early at age nine (Ruban 2005); Chinese Sturgeon mature at ages 8 to 18 years for males and 14 to 26 years for females (Chen et al. 2006); and Shortnose Sturgeon mature at 6 to 10 years for males and 8 to 12 years for females (Department of Energy and Environmental Protection).

Consistent with observations in other sturgeon species (e.g., Flynn and Benfy 2007), gonadal development in Lake Sturgeon begins with an increase in gonadal size largely resulting from a large increase in adipose tissue relative to germinal epithelium. Secondary changes in the germinal epithelium (transitions to pseudostratified epithelia) observed at 15 months in the Shovelnose Sturgeon similarly were visible at 17 months in Lake Sturgeon. However, in contrast to Shovelnose Sturgeon,

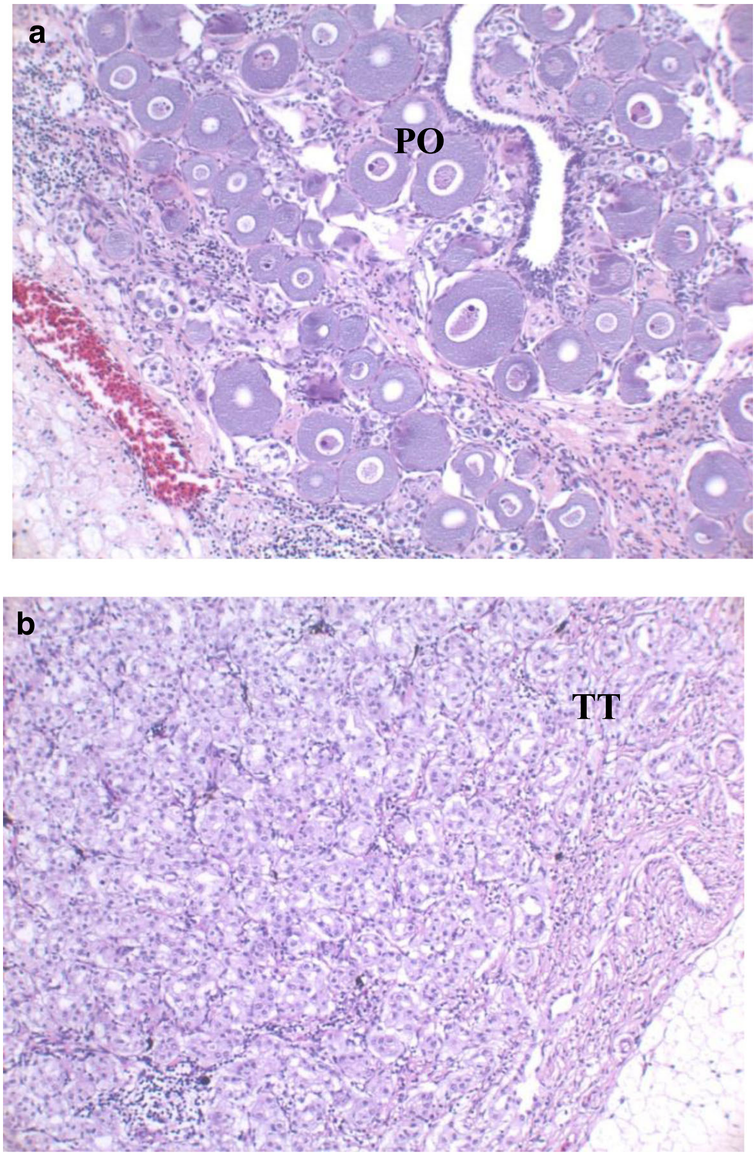


Fig. 9 Gonadal tissue from 53 month old fish under 10X objective showing (**a**) differentiated female gonadal tissue showing primary oocytes (PO), (**b**) differentiated male gonadal tissue showing tubule formation (TT)

where testes and ovaries could be distinguished at 15 months, advanced anatomical differences such as the presence of primordial or primary oocytes were not observed at this time in Lake Sturgeon and gonads were still undifferentiated. Histological differences consistent with sex (presence of prospective primordial oocytes) could not be conclusively assigned until 41 months in Lake Sturgeon, and at 41 months the majority of fish (75%) could not be assigned conclusively to a sex. In contrast, the majority of fish (75%) could be assigned to a sex at 53 months in Lake Sturgeon and provide a more reliable timeframe from which to evaluate sex histologically.

Although male Lake Sturgeon reach sexual maturity earlier than females (Harkness and Dymond 1961; Scott and Crossman 1973; Bruch et al. 2001), gonadal development in males appeared slower than females over the ontogenetic stages evaluated here. At forty-one months, we observed evidence of ova formation in two fish, whereas no males could be conclusively identified at the same age. At fifty-three months, only one male showed evidence of early spermatogenesis and two males showed evidence of tubular and duct formation consistent with mature male samples (Fig. 9b). However, mature spermatagonia were absent. Similarly, in the Adriatic sturgeon, characteristics

Table 1 Lake Sturgeon (*Acipenser fulvescens*) sampled at 53 months

PIT tag number	Total Length (cm)	Mass (g)	Incubation Temperature (10 or 18 °C)	Sex assigned histologically
3671	696	1998	10	Male
7747	711	2031	18	Female
3667	616	1749	18	Male
7760	732	2010	10	Unknown
3655	719	1892	18	Male
7795	730	2093	10	Female
7821	783	2435	18	Female
3688	759	2039	10	Unknown

consistent with female tissue development were observed before male characteristics in early developing juveniles (210 days; Grandi and Chicca 2008), and a similar pattern was observed in Coho salmon where ovarian tissue could be identified at 27 days post hatch, but testicular development occurred several weeks later (Piferrer and Donaldson 1989). Other studies have found similar challenges in sexing immature individuals, particularly males, relative to females (Petochi et al., 2011), and highlight that definitive characteristics for sex-based gonadal development may appear later in males than females and complicate sex assignment at early stages. The disparity between gonadal development and time at maturation is potentially explained by differences in timing of resource allocation to gametes for males compared to females, resulting in gametogenesis beginning sooner but with first reproduction occurring later in females. Relationships between diet quantity and quality can influence gonadal maturation rate in other taxa (e.g., copepods, Chen et al. 2011); however, to our knowledge, studies that identify sex-based differences in timing of gonadal maturation, age at first reproduction, and variation in resource allocation are currently insufficient to evaluate this hypothesis.

Temperature during egg incubation (cold = 10 ± 1 °C and warm = 18 ± 1 °C) was not associated with sex (Table 1), and did not affect overall body size (although females were generally larger than males by 53 months). Fish that could not be assigned a sex were larger than many of our fish that could be assigned sex; however, all size ranges overlapped. Therefore size is an unreliable indictor of sex or the degree of gonadal development at 53 months. In the Black Lake population, differences in body length between cold and warm incubated individuals (both naturally occurring and through hatchery operations) can be substantial (Crossman 2008) and warrants further explanation regarding the timing of gonadal development and relationships to body size.

Lake Sturgeon are a species of conservation concern, and because histological characterization of gonadal tissue required euthanizing the animal, alternative methods for identifying sex will be necessary. Alternatives to sacrificing, such as biopsies of the gonadal tissue, would be difficult, as we found variation in

Table 2 Comparison of age at maturity and timing of histologically-determined sex differentiation of sturgeon species

	Age at maturity (male)	Age at maturity (female)	References	Age at histological sex determination	References
Adriatic Sturgeon	7–11 years	12–14 years	Petochi et al. 2011	20 months	Grandi and Chicca 2008
Lake Sturgeon	12–15 years	18–27 years	Harkness and Dymond 1961; Scott and Crossman 1973; Bruch et al. 2001	53 months	This paper
Shovelnose Sturgeon	4–5 years	6–7 years	Keenlan 199; Jackson 2004	15 months	Colombo et al. 2007
Shortnose Sturgeon	6–10 years	8–12 years	Department of Energy and Environmental Protection	15 months	Flynn and Benfy 2007
White Sturgeon	4 years	8 years	Doroshov 1997	Unknown	Webb et al. 2002

development from anterior to posterior regions of the gonads that is inconsistent with external morphological markers and therefore, would provide an unreliable estimate of sex while risking injury to the organs. Future work should consider validated hormonal assays, ultrasound, or the use of other less invasive (non-destructive) techniques. Hormonal assays have been used as an indicator of sex in Lake Sturgeon and could be a less-invasive way to characterize sex, however challenges remain regarding validation and reliability for using hormones as a predictor of sex in Lake Sturgeon (Craig et al. 2009). Therefore, despite their reliability of use in other sturgeon species (e.g., white sturgeon; Webb et al. 2002), caution must be used when applying hormonal assays for sex-based characterization without histological or other gonadal validation (e.g., Wildhaber et al. 2007; Petochi et al. 2011). Similarly, ultrasound technology is effective for identifying sex and reproductive stage in Lake Sturgeon (Chiotti et al. 2016), but current technology works only among adults and not at the juvenile stage. Validation of sexing methods using histology will be important for identifying the sex determining mechanisms and in guiding future management where stocking is the preferred rehabilitation technique. Validation techniques will require sampling at the earliest possible point where sex can be reliably determined to help to control for sex-specific differences in mortality (Conover 2004), which for Lake Sturgeon, appears to be 53 months.

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