Aquaculture xxx (xxxx) 741735

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Estradiol-17β but not 11β-hydroxyandrostenedione induces sex transdifferentiation in Siberian sturgeon

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ARTICLE INFO

Keywords:
Siberian sturgeon
Sex inversion
Gonadal transdifferentiation
Steroids

ABSTRACT

The Siberian sturgeon (*Acipenser baerii*) is a commercially-important fish. As it is the females that produce the highly-prized caviar, genetic monosex cultures would be valuable. Before such populations can be produced, it will first be necessary to formally confirm whether this species has a ZZ/ZW sex-determination system. This discovery will in turn require the development of successful sex inversion and progeny testing approaches. This work is a first step in advancing this strategy. Our aim was to induce gonadal transdifferentiation using two steroids found naturally in fish —estradiol-17β (E2) and 11β-hydroxyandrostenedione (11β-And)— to feminize and masculinize genetic males and females, respectively.

Steroidal treatments were initiated at 3.5 months of age, using 5 μg/g body weight (b.w.) steroid injections; control fish received vehicle injections. Injections were given every 3 weeks for 7 months, concluding at 10.5 months. Genetic and histological tests were performed at 3.5, 6, 9, and 12 months of age.

All animals remained histologically undifferentiated at 3.5 months. Control females showed signs of sex differentiation at 6 months (columnar epithelium with distinct folds), and at 9 and 12 months, control female and male gonads showed nests of oogonia and scattered spermatogonia, respectively.

The E2 treatment induced male-to-female gonad transdifferentiation in the genetically-sexed males at 6, 9, and 12 months of age. In contrast, 11β-And failed to induce transdifferentiation of the female gonads. While some transient changes were observed, such as increased connective tissue between female germ cell clusters at 9 months, the 12-month-old fish displayed a normal female phenotype with no signs of sex inversion.

Importantly, the experimental E2 treatment produced live phenotypic females transdifferentiated from genetic males, with normal growth, feeding, and mobility as compared with control fish. Further experiments are needed to attempt masculinization of females, potentially with higher doses of natural androgens or other hormones and chemicals.

1. Introduction

The Siberian sturgeon (*Acipenser baerii*) is a commercially-important fish cultivated in 50 countries (Chebanov and Williot, 2018), including Uruguay. Sturgeon farming and caviar production began about 25 years ago in Uruguay, and products including caviar and meat are now exported to the United States, Japan, Europe, and Russia. This species is currently the most commercially-viable aquacultured fish in this country. As it is the females that produce caviar, generating monosex cultures of genetic females could improve profitability. A necessary first step in creating such populations is confirmation of the species' sex-determination system.

The sex-determination mechanism for basal and gonochoric Actinopterygian species such as sturgeon is not fully elucidated (Flynn et al., 2006). In fact, the presence of sex chromosomes has not been demonstrated at the cytological level in sturgeon (Fontana and Colombo, 1974; Van Eenennaam, 1997), possibly because these fish exhibit relatively primitive chromosomal evolution (Volff, 2005). In the absence of formal evidence, various pragmatic approaches have been used to investigate whether sex determination can be explained by chromosomes. Pioneering studies by Van Eenennaam et al. (1999) on sturgeon gynogenesis indicated that the female is likely heterogametic (ZZ/ZW) (Van Eenennaam et al., 1999); however, the authors did not rule out the possibility of an autosome-influenced ZZ/ZA-type system in *Acipenser transmontanus*. All the other work made on gynogenesis in

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https://doi.org/10.1016/j.aquaculture.2024.741735
Received 28 May 2024; Received in revised form 13 September 2024; Accepted 5 October 2024

sturgeons and related species obtained similar sex ratios than those found by Van Eenennaam et al. (1999). Accordingly, the idea that sturgeon sex determination involves a heterogametic ZW female has been generally adopted (Flynn et al., 2006; Fopp-Bayat, 2010; Omoto et al., 2005; Saber and Hallajian, 2014).

The stable 50:50 sex ratio in wild and captive sturgeon populations (Chapman et al., 1996; Ruban et al., 2019; Sanfilippo et al., 2022; Williot et al., 2005; Williot and Brun, 1998) also points to a primary sex-determination factor in Siberian sturgeon, as has been proposed for other fish with similar proportions of males and females (Piferrer, 2001). Finding a sex marker or major sex-determining gene specific to females would be greatly helpful; to this end, geneticists have searched for genetic sex markers in sturgeon for more than 30 years, using a variety of methodologies (Keyvanshokooh and Gharaei, 2010; Khodaparast et al., 2014; Wuertz et al., 2006). Despite massive advances in genomics techniques, such as approaches to detect SNPs associated with sex traits or genes (Du et al., 2020; Kuhl et al., 2021; Lasalle et al., 2022), research has failed to identify any major sex-determining sturgeon genes. A single study has reported a female-specific genome region (Kuhl et al., 2021), a finding that supports the prevailing theory of a ZZ/ZW monofactorial system. However, no formal evidence of such a system has been obtained using hormonal sex inversion or crossbreeding with normal animals. Female Siberian sturgeon cultured in Uruguay mature at 5–6 years of age (Vizziano-Cantonnet et al., 2018a) making a long-term strategy necessary to conclusively evaluate the results of hormonal treatments or progeny experiments. Accordingly, our laboratory is conducting a series of experiments to illuminate the sturgeon sex-determination system. The present study was designed to induce hormonal sex inversion, a crucial first step in advancing this longer-term plan.

The literature on sex inversion in fish is extensive and indicates a pivotal role for steroids in aquacultured fish (Baroiller et al., 1999; Pandian and Sheela, 1995; Piferrer, 2001; Piferrer et al., 1994). Estrogens have been characterized as potent feminizers in fish (Baroiller et al., 1999; Donaldson and Hunter, 1982; Pandian and Sheela, 1995; Piferrer and Guiguen, 2008), and natural estrogens such as estradiol-17β (E2) seem to be better for fish feminization than synthetic options (Piferrer, 2001). Such results are consistent with findings that estrogens are key endogenous regulators of ovarian differentiation in many teleost fish (Guiguen et al., 2010), as well as with the discovery that estrogens activate the female molecular pathway in genetic males when administered to undifferentiated fish (Vizziano-Cantonnet et al., 2008). In the Siberian sturgeon, the early activation of genes involved in estrogen production in future female gonads suggests that female sex differentiation is dependent on endogenous estrogen (Lasalle et al., 2024; Vizziano-Cantonnet et al., 2018a; b), as in many teleost fish (Guiguen et al., 2010). Moreover, E2 is the natural estrogen in sturgeon (Vizziano-Cantonnet, 2018).

Successful sex inversion after E2 treatment has been achieved in several fish, including other sturgeon species (*Acipenser stellatus,* (Falahatkar et al., 2014). Furthermore, an acute injection of 5 μg/g of E2 has been shown to stimulate the female gene *foxl2* in sex-undifferentiated Siberian sturgeon of 2.5 and 3 months of age 12 h after treatment ($p < 0.05$). Since *foxl2* is a key gene for sexual differentiation in teleosts (Baron et al., 2005) and sturgeons (Vizziano-Cantonnet et al., 2018b; Lasalle et al., 2024), we decided to select that dose of E2 to induce sexual transdifferentiation of genetic male Siberian sturgeon.

In terms of androgens, various natural and synthetic hormones have been tested in many species from several fish families, at different time points, doses, durations, and routes of administration. Among the synthetic androgens, 17α-methyltestosterone (MT) is the hormone most widely used to attempt sex inversion of genetic females (Donaldson and Hunter, 1982; Pandian and Sheela, 1995). Nevertheless, results obtained with MT have been variable, with different reported percentages of masculinized, intersex, and sterile fish (Donaldson and Hunter, 1982; Guerrero III, 1975; Pandian and Sheela, 1995). Among natural androgens, testosterone, 11-ketotestosterone (11KT), 11β-hydroxyandrostenedione (11β-And), androstenedione, and dehydroepiandrosterone have successfully induced complete sex inversion in species of at least six families (Pandian and Sheela, 1995). However, some experiments with aromatizable androgens produced a paradoxical feminization, likely through aromatization of exogenous androgens to estrogens (Piferrer, 2001). Despite the high efficiency of natural androgens, they do not seem to be universal endogenous regulators of male differentiation in fish (Blasco et al., 2010; Hattori et al., 2009; Ijiri et al., 2008; Nakamura et al., 1998; Vizziano et al., 2007). In the case of sturgeon, 11KT has been identified by biochemical analysis in the gonads and plasma of adult fish (Vizziano-Cantonnet, 2018); furthermore, non-aromatizable 11-oxigenated androgens could be produced in sexually-undifferentiated gonads, since the gene coding for the enzyme *cyp11c* is expressed during undifferentiated stages (Klopp et al., 2020; Vizziano-Cantonnet et al., 2016, 2018b). Cyp11c is the enzyme that converts aromatizable androgens to 11-oxigenated androgens. In addition, the androgen receptor gene is expressed in the gonads of sex-undifferentiated Siberian sturgeon (Vizziano-Cantonnet et al., 2018b; Vizziano-Cantonnet et al., 2016), suggesting potential androgen sensitivity in undifferentiated gonads. The above background and the successful results obtained using 11β-And in other species (Pandian and Sheela, 1995; Vizziano et al., 2008), led us to select this non-aromatizable natural androgen to induce gonadal transdifferentiation of genetic females into males.

In general, hormonal sex inversion is only exploitable when an effect on the sex ratio can be verified (Baroiller et al., 1999). In Siberian sturgeon, the recent development of a method to identify sex at the genetic level will provide further insight into the efficiency of steroid treatments in gonadal transdifferentiation, and allow us to improve these treatments if necessary.

The gonadal phenotype in gonochoristic species is susceptible to manipulation primarily around the period of sex differentiation (Baroiller et al., 1999), during a "critical window" that corresponds in some species to a molecular sex differentiation period that precedes morphological differentiation (Baroiller et al., 1999; Pandian and Sheela, 1995; Piferrer, 2001; Vizziano et al., 2008; Vizziano-Cantonnet et al., 2008). In previous works, we conducted morphological and molecular studies at early stages of gonadal development and identified a molecular differentiation period from 2.5 to 6 months of age for Siberian sturgeon cultured in Uruguay (Berbejillo et al., 2012, 2013; Lasalle et al., 2024; Vizziano-Cantonnet et al., 2016, 2018a, 2018b).

Based on the above background, we developed a protocol to administer low doses of natural estrogens (E2) and androgens (11β-And) beginning during the molecular differentiation period (3.5 months of age) but prior to morphological sex differentiation.

Our main objective was to achieve a complete sex transdifferentiation of gonads by provoking a process as close as possible to the natural gonadal development process, ultimately producing live, transdifferentiated animals with and adequate growth and feeding behaviors.

2. Materials and methods

2.1. Experimental animals and rearing procedures

Research procedures involving animal experimentation complied with international principles on the use and care of laboratory animals and Uruguayan animal welfare regulations (Comisión Honoraria de Experimentación Animal: CHEA). The protocol was approved by the "Comisión de Etica en el Uso de Animales" of the Comisión Honoraria de Experimentación Animal CHEA of Uruguay (Authorization Number 240011–500874-21). All animals were provided by Estuario del Plata fish farm (Tacuarembó, Uruguay), transported to the aquaculture facilities at the Facultad de Ciencias (Universidad de la República Oriental del Uruguay) at 3 months of age at the natural photoperiod in Uruguay and fed at 2 % of the body weight. The fish were maintained in water at natural temperatures during the spring and autumn (15 to 20 °C), while in summer the temperature fluctuated from 19 to 24 °C, due to a control of temperature in the culture room to prevent high temperatures. They were acclimated for 15 days before treatments were initiated. Length (cm) and weight (g) were measured for all fish sampled. The numbers and characteristics of the fish sampled are shown in Table 1.

2.2. Steroid treatments and animal sampling

Experimental treatments began at 3.5 months of age, which is considered a sex-undifferentiated stage in this species (Rzepkowska and Ostaszewska, 2013; Vizziano-Cantonnet et al., 2016). Prior to initiating the injections, 10 fish were selected at random to confirm that the gonads remained undifferentiated. Steroid injections began immediately after this sampling and were administered every three weeks, for a period of seven months, from 3.5 to 10.5 months of age (see experimental design in Fig. 1). Three mixed-population (males and females) groups were created to monitor changes in gonad morphology at different ages (6, 9, and 12 months): control (Control), estrogen-treated (E2-treated), and androgen-treated (11β-And-treated). The number of control and steroid-treated fish produced and tested at each timepoint are shown in Table 2. Control groups received only saline (0.15 M $NaCl_2$) with 3 % ethanol. The feminizing treatment provided to the E2-treated group consisted of a 5 μg/g b.w. injection of E2 (1,3.5[10]-estratriene-3,17β-diol, SIGMA) (E2) in saline (0.15 M $NaCl_2$) with 3 % ethanol; while the masculinizing treatment given to the 11β-And-treated group consisted of a 5 μg/g b.w. injection of 11β-hydroxyandrostenedione (4-androsten-11β-ol-3,17-dione, SIGMA-ALDRICH, St. Louis, MO) (11β-And) in saline (0.15 M $NaCl_2$) with 3 % ethanol.

Steroid treatment concluded at 10.5 months of age, after which fish were maintained until 12 months of age. Sampling was performed to study gonad morphology at 3.5 months of age (before the first injection), and at 6, 9, and 12 months of age (during and after hormone treatment) (Fig. 1). Gonad samples for histology were preserved in Bouin-Holland (Gabe, 1968) for 24 h and then washed two times in ethanol 70:30 v:v and stored until use. For sexing, the caudal fin was sampled and conserved in ethanol 95:5 v:v for later DNA extraction. Sampling for gene expression was performed in fish at 6 months of age under sterile conditions, and gonads were extracted and conserved in liquid nitrogen (N_2) to prevent degradation. After sampling, tissues were stored at −80 °C until RNA extraction.

2.3. Histological analyses

Fixed gonads were dehydrated, embedded in paraffin, cut into 5-μm-thick sections, and stained with Mayer's hematoxylin-eosin and Harris hematoxylin-eosin.

(Gabe, 1968). Stained tissue sections were observed with an Olympus Provis microscope and imaged with an Olympus DP50 camera using Cell software.

2.4. Genomic DNA extraction and qualitative PCR for sexing

Caudal fin samples were collected in sterile 1.5 ml microcentrifuge tubes. A.

small portion (2 mm) was placed in a microcentrifuge tube. Then, 25 μl of tissue.

digestion buffer (NaOH 25 mM and EDTA 0.2 mM) was added and briefly vortexed. Each.

sample was heated for 15 min at 95 °C in a thermocycler BIOER (LifeECO). Samples were then briefly vortexed and spun. Following digestion, 25 μl of neutralization buffer.

(Tris buffer 40 mM, pH 8) was added. Isolated DNA samples were stored in.

microcentrifuge tubes at −20 °C.

The AllWSex2 primers published by Kuhl et al. (2021) were used to sex the fish. gDNA samples were amplified by qualitative PCR and validated by inspection on 2 % agarose gel. Each reaction was constituted by 0.312 μl of 10 μM of forward and reverse primers, 1 μl of gDNA (20 ng/μl), 0.25 μl of NZYTaq II DNA polymerase (NZYTech), 2.5 μl of 10× reaction buffer, 1.25 μl of MgCl 2 50 mM, 3.75 μl of 2 mM dNTP mix (Thermo Fisher Scientific, Waltham, MA), and water, for 25 μl of total volume. The run method performed was: 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and 72 °C for 8 min.

Table 1
Fish characteristics by age (SD: standard deviation).

Gene	Forward primer	Reverse primer	Product (bp)
cyp19a1	5´ GTCCAGCTTCAGACAGGATG 3´	5´ AGATGGTGTCGTGCAATGTT 3´	164
foxl2	5´ GAGGCCTTTCAGACCCCCTCC 3´	5´ TGGGCTGACGTTGCCACTT 3´	189
rpl8	5´ TCTGAGACGGTCTTTGTTCC 3´	5´ GGTAACCATCAGCACATTGG 3´	122
AllWsex2	5´ TGATCAACCTCTTCAGCAATGTC 3´	5´ TGAGAGCCACTGTACTAACACA 3´	108

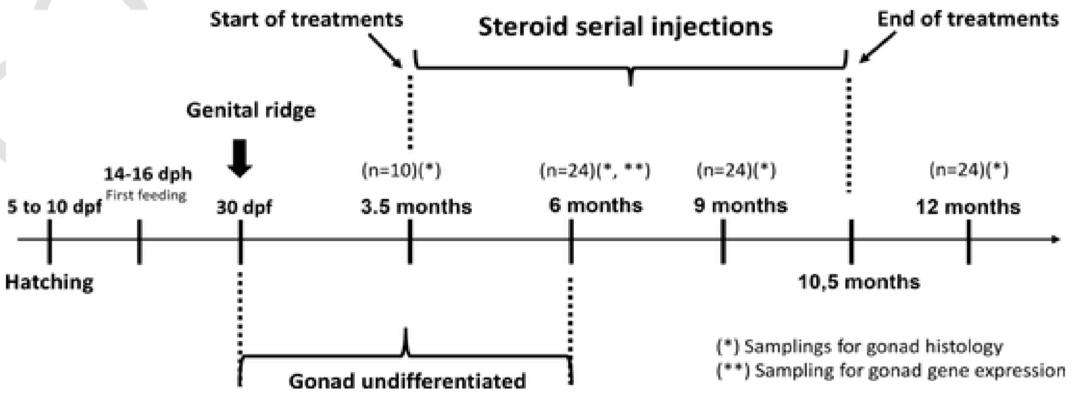


Fig. 1. Experimental design and sample collection.

Table 2
Number of fish studied at each stage for control and steroid-treated *A. baerii* groups.

Age (months)	n	Average length (cm) ± SD	Average weight (g) ± SD
3.5	10	12.94 ± 1.7	7.4 ± 2.3
6	24	28.2 ± 3.6	58.5 ± 22.4
9	24	38.6 ± 4.9	160 ± 66
12	24	46 ± 5	295 ± 103

2.5. RNA extraction and reverse transcription

Total RNA was extracted using the Monarch Total RNA Miniprep kit (New England BioLabs Inc., MA) according to manufacturer instructions. RNA integrity was validated using a 2 % agarose gel electrophoresis, loaded with 700 ng of pure RNA. The presence of genomic DNA was verified after RNA purification by PCR, performed on the RNA extracted using primers for rpl8. Samples with contamination of genomic DNA were treated with DNase I, Amplification Grade (Invitrogen, Carlsbad, CA) and checked again by PCR after the DNase treatment. cDNA synthesis was carried out using 1 μg of total RNA in a 20-μl volume reaction. RNA was denatured in the presence of 200 ng of random primers and 0.5 mM of dNTP for 5 min at 65 °C and then chilled on ice. Reverse transcription was performed at 37 °C for 50 min using M-MLV RT (Invitrogen, Carlsbad, CA) according to manufacturer instructions. The cDNA samples obtained were used to analyze expression of selected genes by qPCR.

2.6. Quantitative PCR (qPCR)

As the female-to-male sex inversion with the androgen treatment failed, expression of two essential female genes known to be repressed by androgens (*cyp19a1, foxl2*) (Vizziano et al., 2008) was studied in control and androgen-treated females.

Primers for qPCR analysis of *cyp19a1* and *foxl2* were designed with the specific gene sequence obtained in the transcriptome study (Klopp et al., 2020; Vizziano-Cantonnet et al., 2018b) and previously validated by melting curve analysis (qPCR), agarose gel electrophoresis, and sequencing (Lasalle et al., 2021). The primers used are shown in Table 3. Real-time PCR experiments were carried out on an Applied Biosystems instrument (ABI 7500) using 96-well plates. Reactions were performed in a 12-μl total volume. Each reaction was constituted by 200 of nM forward and reverse primers, Power Up SYBR Green qPCR Master Mix, and 10 ng cDNA for target genes or 3.3 ng of cDNA for the housekeeping gene. The run method performed was a holding stage at 50 °C for 2 min and 95 °C for another 2 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and finally the melting curve stage, at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The validity of the qPCR experiment was confirmed by checking the melting curves, melting temperatures of the samples, and negative controls. A standard curve was developed for the assay with each gene. The efficiencies of primers used are shown in the Table 2.

Real-time PCR efficiencies (E) and systemizing quotients (SQ) for the reactions were calculated using the formulas: E = [10ˆ(−1/−slope)] and SQ = [10ˆ((Ct-intercept)/slope)]$^{-1}$ (Baron et al., 2005). Relative gene expression was measured by dividing the SQ of the target gene by the SQ of the reference gene (*rpl8*), which was validated for undifferentiated and differentiated gonads in previous studies (Berbejillo et al., 2012; Vizziano-Cantonnet et al., 2016).

Table 3
Primer set used for gene expression using real-time polymerase chain reaction (PCR) and for sexing fish with end-point PCR.

Age (months)	Control	E2-treated	11β-And-treated
3.5	10	–	–
6	8	8	8
9	8	8	8
12	9	8	8

2.7. Statistical analysis

Normality and homogeneity of variance assumptions were evaluated for the weight and length data. For each developmental stage studied after treatment was initiated (6, 9, 12 months of age), the weights and lengths of control, E2-treated, and 11β-And-treated fish were compared using ANOVA when the assumptions of normality and homogeneity of variance were not rejected (Sokal and Rohlf, 1995), or Kruskal-Wallis tests when those assumptions were rejected. Normality of data and homogeneity of variance assumptions were evaluated for each gene at 6 months of age. T-student was used for comparing gene expression between sexes when the assumptions of normality and homogeneity of variance were not rejected. Mann Whitney tests were used when those assumptions were rejected.

3. Results

3.1. Effects of treatments on weight and length

Experimental fish survived to the end of the experiment at 12 months of age. Under both treatments (E2 and 11β-And), the experimental fish fed well and remained active as compared to control fish. The average ± standard deviation for the weights and lengths of control, E2-treated, and 11β-And-treated fish at 6, 9, and 12 months of age are shown in Fig. 2. The treatments did not induce significant changes in weight or length ($p > 0.05$) at 6, 9, and 12 months of age.

3.2. Natural gonad morphology in sexed fish

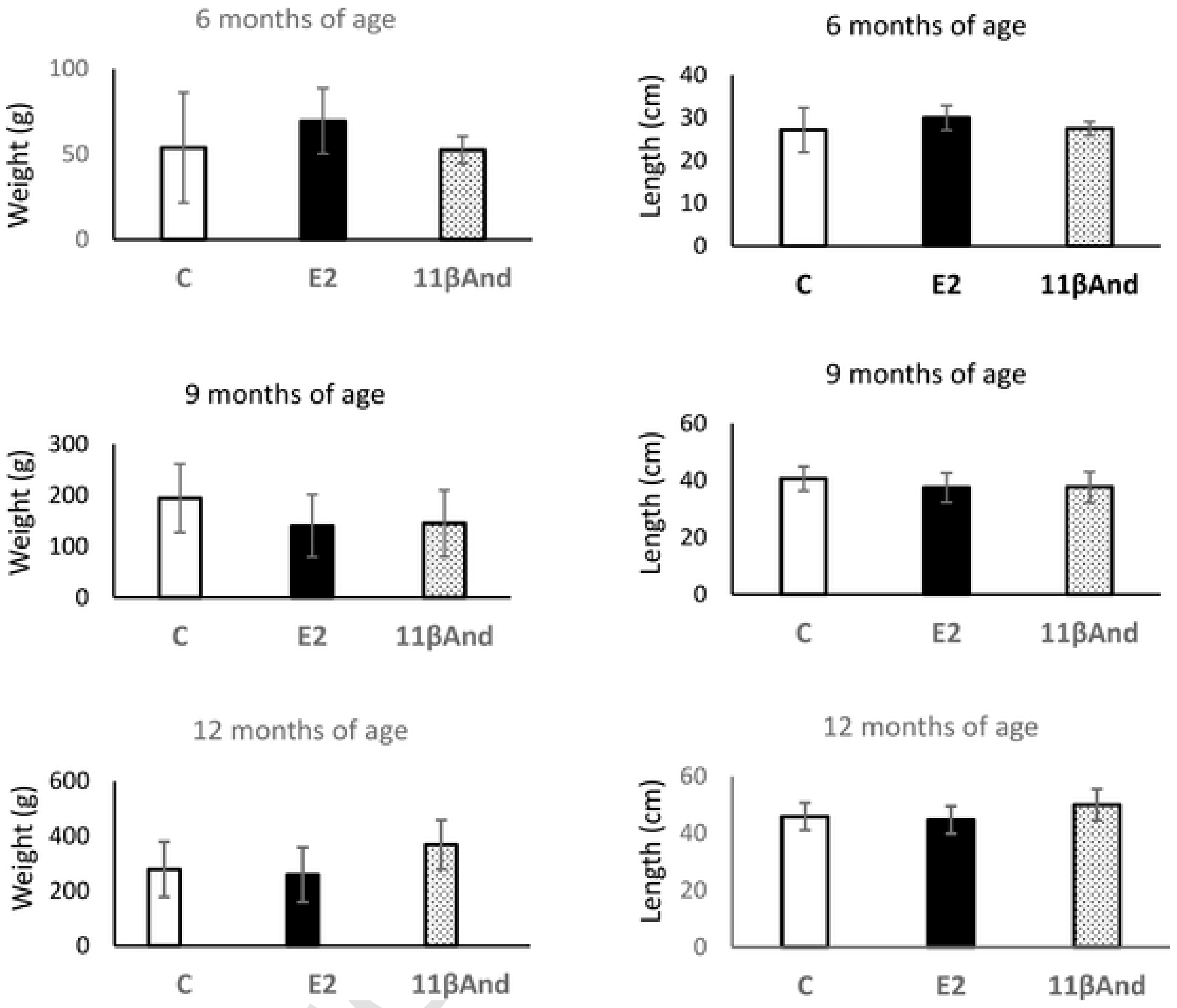
Sampling was carried out at 3.5, 6, 9, and 12 months of age to assess the morphological development in the gonads of the control fish. Males and females were genetically sexed at all periods studied.

At 3.5 months of age, the gonads of genetically-sexed males ($n = 4$) and genetically-sexed females ($n = 6$) showed no signs of sex differentiation. At this stage, one side of the gonad was covered with an epithelium constituted by columnar or cuboidal cells, while the other side showed a flat epithelium (Fig. 3A). Connective tissue was evident inside the gonad. Primordial germ cells were restricted to the periphery among epithelial cells, or located inside the gonads surrounded by somatic cells, observed both at the epithelium level and inside the connective tissue (Fig. 3B). The gonads of control female (Fig. 4A) and male (Fig. 4B) fish were undistinguishable.

At 6 months of age, the gonads of genetically-sexed females ($n = 5$) showed recognizable signs of sex differentiation as compared to genetically-sexed males ($n = 3$). The gonadal area of all females studied was covered with a columnar epithelium with clear folds (Fig. 4C). In testes, the surface remained smooth and the epithelium cuboidal, surrounding well-developed lax connective tissue (Fig. 4D). Germ cells were located both in the epithelium and the connective tissue (Fig. 4C, D).

At 9 months of age, the ovaries of all genetically-sexed control females ($n = 3$) showed the classic signs of ovarian development with clusters or nests of meiotic germ cells, corresponding to the first stages of oocyte development, and isolated primary growth oocytes surrounded by epithelial cells (Fig. 4E). The testes of all genetically-sexed control males ($n = 5$) showed a more developed gonad than at 6 months of age, with a smooth surface, columnar or cuboidal epithelium, and many germ cells within the gonad, surrounded by pre-Sertoli cells and connective tissue (Fig. 4F).

At 12 months of age, the gonads of genetic females ($n = 6$) showed ovigerous lamellae with clusters of meiotic germ cells (Fig. 4G), and the gonads of genetic males ($n = 3$) conserved similar characteristics as at 9 months (Fig. 4H).


6 months of age

6 months of age

9 months of age

9 months of age

12 months of age

12 months of age

Fig. 2. Total length and weight of control (C), E2-treated (E2), and 11β-And-treated fish at 6, 9, and 12 months. There were no significant differences in weight or length ($p > 0.05$) between treatments at each age studied.

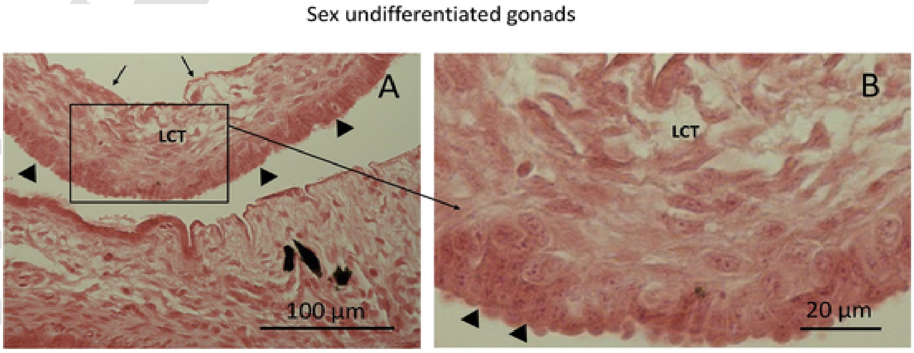

Sex undifferentiated gonads

Fig. 3. Morphology of sex-undifferentiated *A. baerii* gonads at 3.5 months of age. A) Arrows = flat epithelium; arrowheads = columnar epithelium; LCT: lax connective tissue. B: Detail of A showing LCT: lax connective tissue.

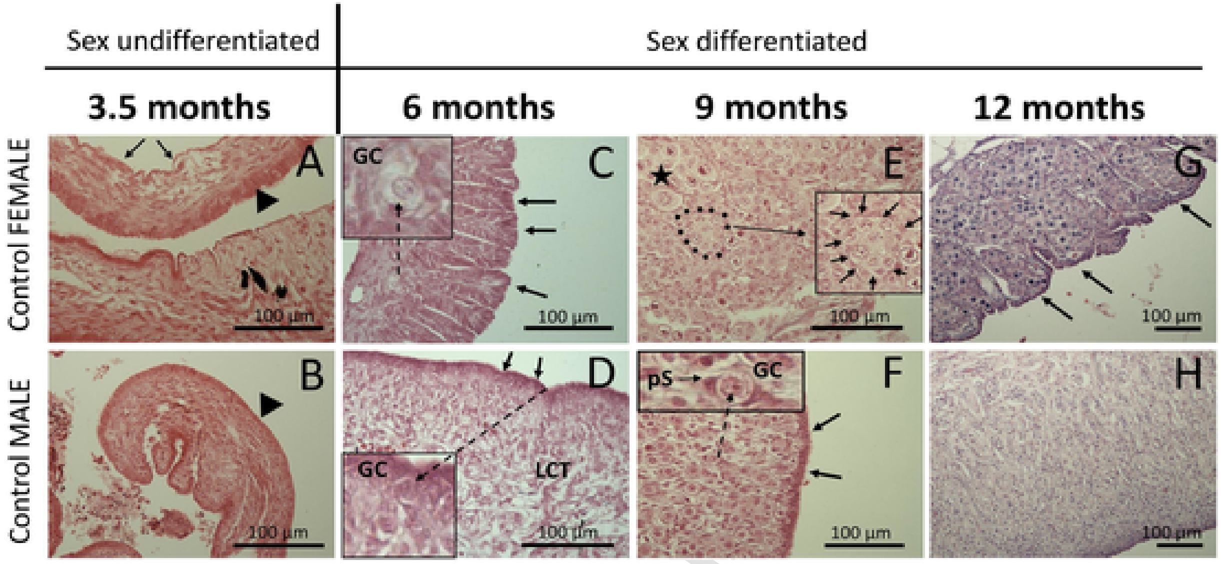


Fig. 4. Morphological changes observed during gonadal development of genetically-sexed *A. baerii* females and males. A: Female gonad at 3.5 months of age; arrows = flat epithelium; arrowheads = columnar epithelium. B: Male gonad at 3.5 months of age; arrowheads = columnar epithelium. C: Female gonad at 6 months of age; arrows = columnar epithelium with folds; GC = germinal cell. D: Male gonad at 6 months of age; arrows = smooth surface of the gonad; LCT = lax connective tissue; GC = germinal cell. E: Female gonad at 9 months of age; dots = cluster of female meiotic germ cells; star = primary-growth oocyte; arrows = magnification of a cluster of female meiotic germ cells. F: Male gonad at 9 months of age; arrows = smooth surface of gonads with cubic epithelium; dotted arrow = magnification of a spermatogonia; pS = pre-Sertoli cell. G: Female gonad at 12 months of age; arrows = ovigerous lamellae filled with meiotic germ cells. H: Male gonad at 12 months of age.

3.3. Effects of E2 treatment on gonad morphology

Fig. 5 provides a comparison of the gonadal morphology of three groups: genetically-sexed females undergoing natural development, genetically-sexed males undergoing natural development, and genetically-sexed males subjected to feminizing treatments.

By 6 months of age, the fish had received 2.5 months of E2 treatment. Clear signs of gonad transdifferentiation into the female phenotype were observed in all genetically-sexed males studied (n = 3) (Fig. 5). The E2-treated gonads of the genetically-sexed males (Fig. 5B) showed the same morphological characteristics as the ovaries of genetically-sexed control females (Fig. 5A). The transdifferentiated gonads of genetically-sexed males treated with E2 differed from the testes of control males and indeed could not be distinguished from natural ovaries at this stage (Fig. 5C). The natural testes had a smooth surface, whereas the feminized males displayed a columnar gonad epithelium with clear folds.

At 9 months of age, after the animals had received 5.5 months of treatment, the gonads of E2-treated genetic males (n = 3) showed clear signs of feminization. In fact, large areas of meiotic germ cell clusters, typical of an ovary, were observed in all gonads of E2-treated males (Fig. 5E), similar to the normal ovaries at this age (Fig. 5D). These E2-treated male gonads were clearly distinguishable from those of control males at the same age, in which non-meiotic germ cells were homogeneously distributed inside the gonad (Fig. 5F).

By 12 months of age, the estrogen treatment had concluded 1.5 months prior. The gonads of E2-treated genetic-sexed males (n = 6) showed clear ovigerous lamellae (Fig. 5H) that were more developed than in control females (Fig. 5G). Inside the lamellae, clusters of meiotic germ cells could be observed, along with primary-growth oocytes (Fig. 5H). In 4 of the 6 E2-treated males, the ovary was more developed than in the control females and showed several primary-growth oocytes.

3.4. Effect of 11β-and treatments on gonad morphology

Figs. 6, illustrate the gonad morphology of the genetic females subjected to masculinizing 11β-And-treatments at 6, 9, and 12 months of age as compared to the natural development observed in genetically female and male Siberian sturgeon in the control group.

At 6 months, the animals had received 2.5 months of 11β-And treatment. As shown, the treatment failed to induce a clear male gonad in the treated genetic females (n = 3). In two cases, the gonadal surface of androgen-treated genetic females conserved the columnar epithelium with the distinct fold characteristic of the control female gonad (Figs. 6B) but also developed a lax connective tissue characteristic of newly-differentiated male gonads. However, neither of these two androgen-treated gonads had acquired the classical characteristics of a male gonad at this stage. In a third case, androgen treatment seemed to accelerate the development of germ cell clusters (data not shown), similar to which were observed in the control female gonads later, at 9 months of age (Fig. 4E).

By 9 months of age, the animals had received 5.5 months of 11β-And treatment. Of the three androgen-treated females studied, one maintained all of the characteristics of a normal female with a columnar epithelium and clusters of meiotic germ cells (Fig. 6E and 7A). A second gonad showed some signs of disorganization as compared to a normal female gonad, including connective tissue between the germ cell clusters (Fig. 7B). The third fish showed significant signs of disorganization, with some germ cell remnants and large amounts of connective tissue (Fig. 7C).

The fish were finally evaluated at 12 months of age, 1.5 months following the conclusion of the androgen treatment. Each of the three females displayed different stages of development after androgen treatment: the first had characteristics similar to those of control females with visible ovigerous lamellae and clusters of meiotic germ cells (Fig. 8A); the second had ovigerous lamellae, clusters of meiotic germ cells, and several primary-growth oocytes (Fig. 8B); and third showed accel-

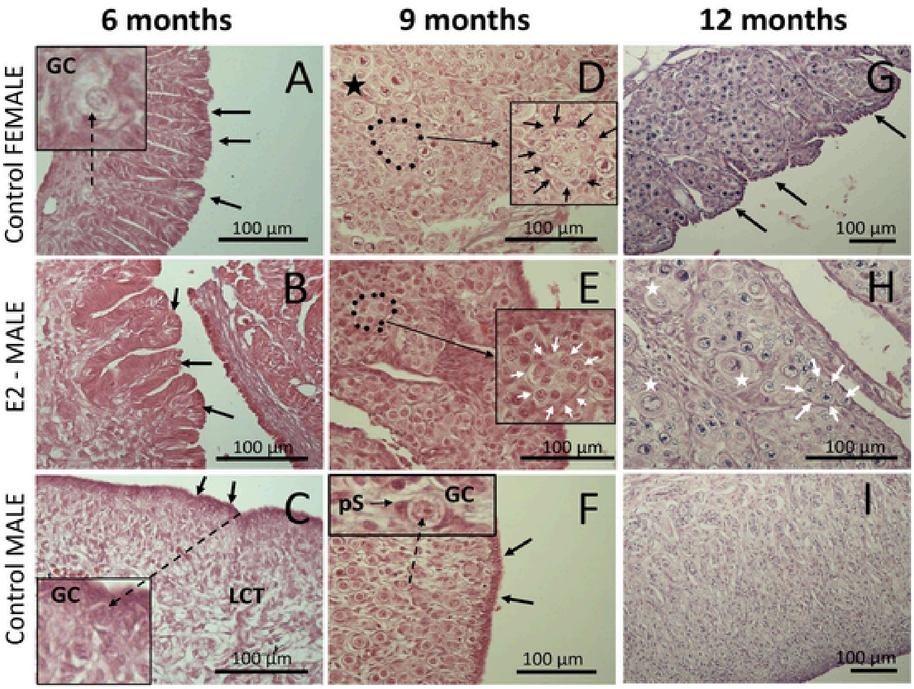


Fig. 5. Comparison of the morphological changes observed during gonadal development of genetically-sexed *A. baerii* females and males, during and after E2 treatment of genetically-sexed males, at 6, 9, and 12 months of age. A: Female gonad at 6 months of age; arrows = columnar epithelium with folds. B: Gonad of a genetically-sexed male at 6 months of age after E2 treatment; arrows = columnar epithelium with folds. C: Male gonad at 6 months of age; arrows = smooth surface of the gonad; LCT = lax connective tissue. D: Female gonad at 9 months of age. Dots = cluster of meiotic germ cells; star = primary-growth oocyte; arrows = magnification of a cluster of meiotic germ cells. E: Gonad of a genetically-sexed male at 9 months of age after E2 treatment. Dots = cluster of meiotic germ cells; arrows = magnification of a cluster of female meiotic germ cells. F: Male gonad at 9 months of age; arrows = smooth surface of gonads with cubic epithelium; dotted arrow = magnification of a spermatogonia. Star = pre-Sertoli cell. G: Female gonad at 12 months of age; arrows = ovigerous lamellae filled with meiotic germ cells. H: Gonad of a genetically-sexed male of after E2 treatment; arrows = boundary of the ovigerous lamellae; star = primary-growth oocytes. I: Male gonad at 12 months of age.

erated ovarian development with large numbers of primary-growth oocytes (Fig. 8C).

3.5. Gene expression in 11β-and-treated fish

cyp19a1 and *foxl2* expression were assessed in control and androgen-treated females at 6 months of age. There were no statistical differences in gene expression between control and androgen-treated genetic females ($p > 0.05$) (Fig. 9).

3.6. Critical window for sex inversion

Based on the results of the present work, we propose that the labile period during which fish are susceptible to E2-induced sex transdifferentiation from genetic males into phenotypic females corresponds to the stage in which the gonads remain undifferentiated (Fig. 10). However, the complete "critical window" of sensitivity to sex change in this species was not assessed.

4. Discussion

In the present work, we describe a sex-inversion protocol in Siberian sturgeon using natural estrogens. The experimental conditions produced live, fully male-to-female transdifferentiated fish with healthy growth and feeding behaviors. These sex-inverted animals obtained will be used in future experiments to further elucidate the sex-determination system used by the species. Specifically, the transdifferentiated specimens will be crossed with normal males and the resulting progeny evaluated. These results also provide the groundwork for further E2-feminization treatment studies with larger numbers of Siberian sturgeon.

In contrast to the estrogen treatment, the natural 11-oxigenated androgen 11β-And did not induce female-to-male sex transdifferentiation at the experimental dosage, treatment duration, and stage. Below, we discuss the natural morphological development observed in genetically-sexed control fish as well as the results of the steroidal treatments in the context of existing data in other sturgeon species.

4.1. Sex differentiation in control fish

Before the possibility of genetic sexing existed, morphological changes were the earliest available indicators of sex differentiation in sturgeon. The major early sign of female differentiation is the presence of nests the oogonia in ovaries, which are extensions of the germinal epithelium called "sex cords" (Grier et al., 2016). Based on this criterion, the Siberian sturgeon was considered sex differentiated at 6.7 months of age in Poland (Rzepkowska and Ostaszewska, 2013) and at 8 months in Uruguay (Vizziano-Cantonnet et al., 2016, 2018a); and Adriatic sturgeon *Acipenser naccarii* was reported to be differentiated at 13.7 months (Grandi and Chicca, 2008). In setting up the present experiment, we defined the undifferentiated period as the time between one month of age, when genital ridge formation occurs (Rzepkowska and Ostaszewska, 2013), and 6 months of age, when the first signs of morphological differentiation were not already evident (Vizziano-Cantonnet et al., 2016, 2018a). We therefore chose to initiate hormonal treatments at 3.5 months of age, during a morphologically-undifferentiated period within the molecular sex differentiation period (Vizziano-Cantonnet et al., 2016), based on prior evidence that the best time to induce a sex

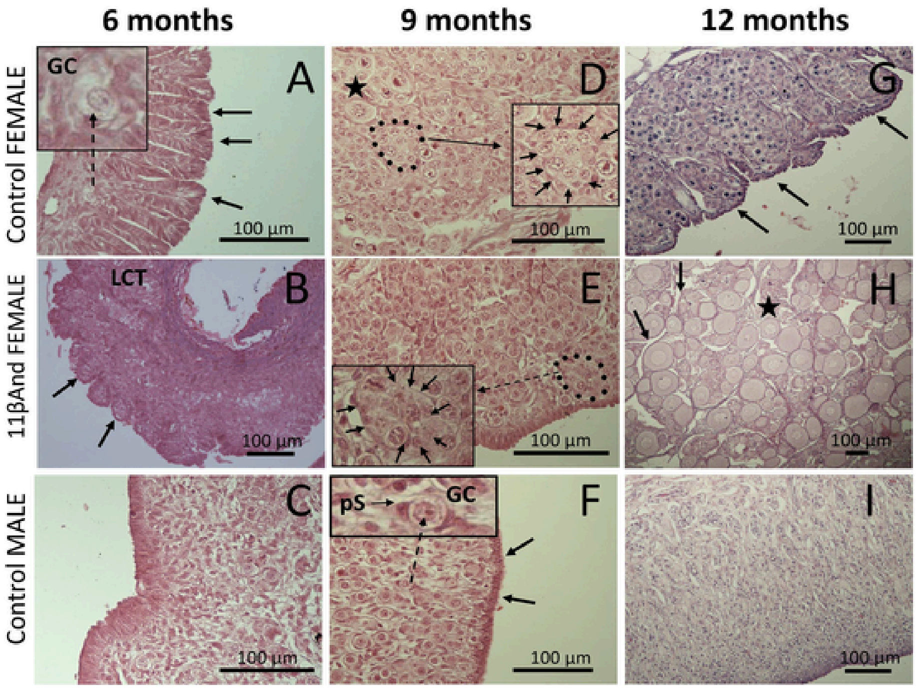


Fig. 6. Comparison of the morphological changes observed during gonadal development of genetically-sexed *A. baerii* females and males, during 11β-And treatment of genetically-sexed females, at 6 months of age. Control FEMALE: arrows = columnar epithelium with folds. Control MALE: arrows = smooth surface of gonads with cubic epithelium; LCT = lax connective tissue. A to C: Gonad of a genetically-sexed female at 6 months of age after 11β-And treatment. In A and B: arrows = columnar epithelium with folds; LCT = lax connective tissue. C: Arrows = cluster of meiotic germ cells.

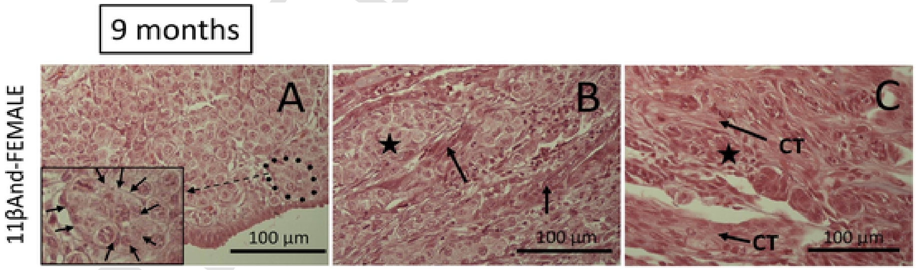


Fig. 7. Morphological changes observed in gonadal development during 11β-And treatment of genetically-sexed *A. baerii* females, at 9 months of age. A: Dots = cluster of female meiotic germ cells; arrows = magnification of a cluster of female meiotic germ cells. B: Star = cluster of female meiotic germ cells; arrows = connective tissue. C: Star = disintegrating cluster of meiotic germ cells; CT = connective tissue.

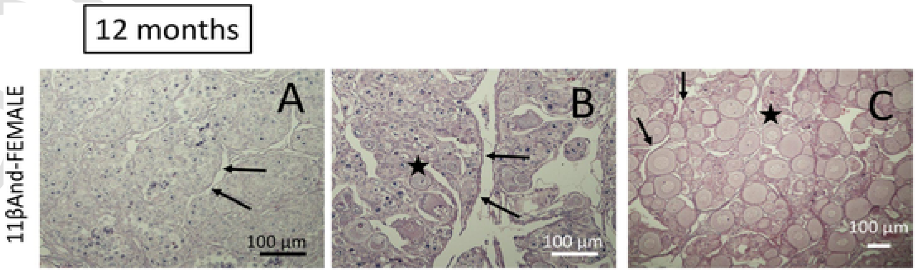


Fig. 8. Morphological changes observed in gonadal development during 11β-And treatment of genetically-sexed *A. baerii* females, at 12 months of age. A – C: Ovaries with ovigerous lamellae; arrows = boundary of ovigerous lamellae; (star) primary growth oocytes.

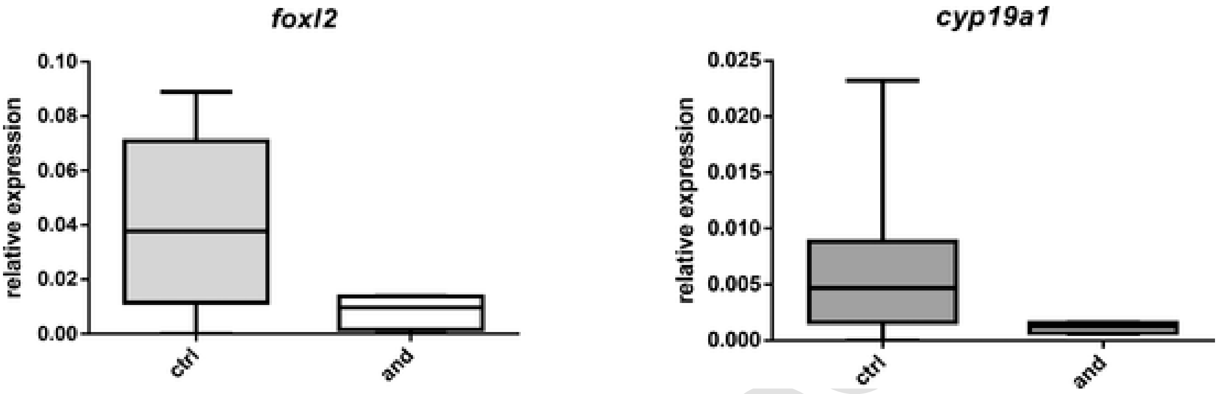


Fig. 9. Gene expression in genetically-sexed control females (ctrl) and genetically-sexed females treated with 11β-And. No significant differences were found ($p > 0.05$).

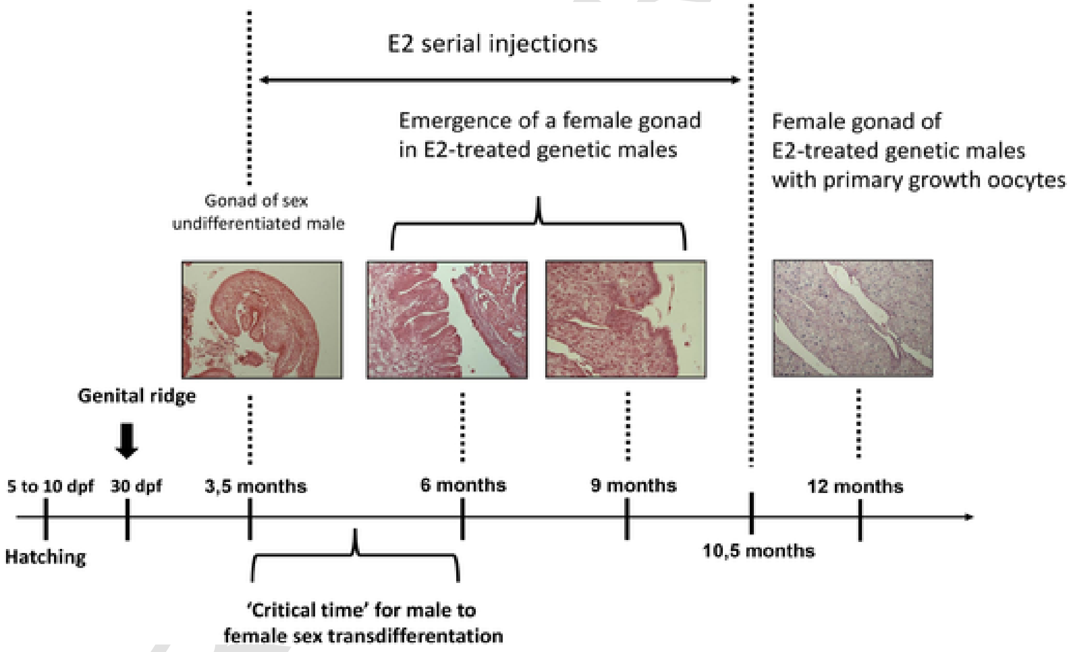


Fig. 10. Schematic representation of gonadal development after E2 treatment and identification of critical time for male sex transdifferentiation.

change hormonally is prior to morphological differentiation (Pandian and Sheela, 1995; Baroiller et al., 1999). To validate this design, we studied the gonadal morphology of genetically-sexed sturgeon and confirmed that the Siberian sturgeon gonads used in the present work remained undifferentiated at 3.5 months of age, consistent with descriptions reported in various sturgeon species (*A. naccarii,* Grandi and Chicca, 2008; *A. gueldenstaedtii, A. baerii*, Rzepkowska and Ostaszewska, 2013; *A. baerii*, Vizziano-Cantonnet et al., 2016).

We also expected that the gonads would remain undifferentiated at 6 months of age based on previous studies in the species (Rzepkowska et al., 2014; Vizziano-Cantonnet et al., 2016, 2018a). However, the gonads of the genetically-sexed female fish showed the presence of a columnar epithelium with distinct notches and folds at 6 months. This morphological criterion had been used to identify "putative ovaries" of Siberian and Russian sturgeon (Rzepkowska and Ostaszewska, 2013), Adriatic sturgeon (*A. naccarii*) (Grandi and Chicca, 2008), and the hybrid bester (Omoto et al., 2001) before genetic sex markers were available. The genetic sexing data collected here supports the idea that this feature is a good morphological reference to distinguish a future ovary from a future testis in Siberian sturgeon. Histology confirmed the first signs of differentiation in the female gonads.

These results underscore the importance of genetic sexing in defining the major stages of sexual differentiation: a) the period in which only molecular differences can be observed (Vizziano et al., 2007); versus b) the gonadal differentiation period from the first morphological sign of differentiation to the structuration of an immature gonad (Yamamoto and Luckenbach, 2024).

The findings prompt us reconsider the molecular sex differentiation period of Siberian sturgeon, previously defined as up to 6 months of age (Lasalle et al., 2024; Vizziano-Cantonnet et al., 2018a). The timing of the first morphological signs of sexual differentiation in the Siberian sturgeon should be further studied using genetically-sexed fish 3.5–6 months of age. The histological descriptions of the control gonads were used as a reference to study the effects of steroidal treatments on gonad structure.

4.2. *Sex inversion after steroid treatments*

The following sections discuss the natural estrogen (E2) and androgen (11β-And) treatments used to attempt transdifferentiation of males and females, including the experimental dosage, duration, and timing.

4.2.1. *Hormonal feminization*

In sturgeon, scarce sex reversal experiments have been carried out using estrogens, and diverse results have emerged depending on dosage, form of administration, and gonadal stage at which treatment was initiated. Orally-administered E2 has been used in several sturgeon species: the shortnose sturgeon *Acipenser brevirostrum*, the hybrid sturgeon bester (*Huso huso* female x *Acipenser ruthenus* male), the beluga (*Huso huso*), and the Persian sturgeon (*Acipenser persicus*), at doses from 10 to 1000 mg/kg b.w. diet and at various different stages of development.

In the shortnose sturgeon, 10, 50, or 100 mg/kg b.w. diet all induced feminization, whether applied during the undifferentiated stage (5 months of age) or early differentiation (7 months). The authors observed that the final sampling at 9 months "revealed that all treated fish were apparently feminized" (Flynn and Benfey, 2007).

In bester, high doses of E2 at 1000 mg/kg b.w. diet during the undifferentiated period (3-month-old fish) successfully feminized fish by 9 months of age, as part of a protocol in which E2 was provided for a total of 15 months (Omoto et al., 2002). The morphological criterion used to identify gonadal sex was based on the gonadal surface. However, gonads with folds can be also observed in intersex gonads (Rzepkowska et al., 2014); thus, the gonadal surface alone is not a good criterion to identify future ovaries. In the same species, high concentrations of E2 (10 μg/g b.w. diet) administered to sex-differentiated fish 14–31 months of age resulted in an incomplete feminization. The authors described developing oocytes in gonads without ovarian lamellae, with an overall structure that more closely resembled a normal testis than an ovary (Omoto et al., 2002). In other words, E2 administered to sex-differentiated fish were feminized the germ cells but not the gonadal structure.

In the case of the beluga and the Persian sturgeon, oral administration of 30 mg/kg b.w. for 40 or 100 days impaired growth performance and failed to change the sex of the fish. Treatment was initiated at 172 g (beluga) and 45 g (Persian sturgeon); as body weight alone is not indicative of the stage of gonad development (Agh and Irani, 2019), it cannot be used to determine the treatment began during the undifferentiated stage.

The only protocol reported to induce effective feminization accompanied by normal growth was carried out in stellate sturgeon (*A. stellatus*), with long-term E2 injections (5 mg/kg b.w.) administered for 6.3 months beginning at 5 months of age (Falahatkar et al., 2014). At the time the study was performed, sex markers were not available, and the authors only describe the gonad morphology at end of the experiments. Although the morphological sex differentiation period in the stellate sturgeon is undocumented, it is likely that the gonads remained undifferentiated or were just beginning to show signs of sex differentiation at 5 months.

In the present work, we followed a strategy similar to the stellate sturgeon protocol described above, but we monitored gonadal changes in the genetically-sexed fish during treatment to follow the processes of natural differentiation and hormonal-induced transdifferentiation. The relatively low doses of E2 injected periodically (5 mg/kg b.w.) in genetically-sexed male Siberian sturgeon during the sexually-undifferentiated stage (3.5 months of age) produced remarkable results. In fact, E2 induced a change in the overall structure of the Siberian sturgeon gonad of genetic males, generating folds and a typical ovarian structure by 6 months of age after 2.5 months of treatment. The treatment also induced the differentiation of primordial germ cells towards clustered oogonia and primary-growth oocytes, which emerged clearly by 9 and 12 months of age, respectively. These global changes in structure were not observed during gonadal transdifferentiation of trout, in which primary-growth oocytes have been observed in gonads without ovigerous lamellae (Vizziano-Cantonnet et al., 2008). Thus, estrogens administered at the correct dosage and timing can likely directly or indirectly regulate several genes that drive not only female germ cell differentiation, but also the development of ovarian structure. In fact, a transcriptomic work performed using undifferentiated Siberian sturgeon gonads demonstrated an upregulation of several genes related to gonad structuration two months after the activation of genes related to estrogen production in females (Vizziano-Cantonnet et al., 2018b). It is likely that some of these genes related to gonad structuration are dependent on estrogens. In the present work, E2 treatment induced the presence of primary-growth oocytes in some fish, suggesting that E2 drives the progression of meiotic germ cells in clusters to isolated primary-growth oocytes as has been proposed in salmon (Campbell et al., 2006). However, this effect needs to be confirmed using larger numbers of fish.

Another interesting feature is that the gonadal structure was similar in transdifferentiated and control fish at 6 and 9 months. This results supports the idea that E2 provokes a response in the gonads of genetic males similar to that produced physiologically by estrogens in the natural female sex differentiation process, as described in other fish (Guiguen et al., 2010). We believe that the more similar the induced sex change to natural development, the better the chances of achieving satisfactory development in the treated individuals.

Global data from various sturgeon species suggests that the critical period during which the gonad is sensitive to E2-induced sexual transdifferentiation corresponds to the undifferentiated stage. However, there are exceptions. At least in the hybrid sturgeon bester, treatment after gonad differentiation induced a sex change in germ cells (Omoto et al., 2002), confirming the high plasticity of the germ line and its capacity for sexual inversion even in already sex-differentiated sturgeon gonads. This high sensitivity to transdifferentiation during immature stages may explain the reported presence of intersex sturgeon (Rzepkowska et al., 2014).

It is difficult to compare orally-administered doses with those given by injection as the estrogen availability in each case likely varies and cannot be easily measured. However, the treatment used in the present work, albeit in a small number of fish, was successful in at least two types of sturgeon (stellate sturgeon, Falahatkar et al., 2014; Siberian sturgeon, present work) and will potentially be useful in other sturgeon species.

We propose that a specific hormonal protocol, in terms of dosage, duration, and timing, may be required induce complete male-to-female gonad inversion in Siberian sturgeon. The success of this treatment confirms that an estrogen-rich environment sustains the ovarian development pathway during the early stages of Siberian sturgeon development until the immature stage, as has been observed in teleost fish (Piferrer, 2001).

4.2.2. *Hormonal masculinization*

In sturgeon, the few existing reports on gonad masculinization all used oral administration of the synthetic androgen 17α-methyltestosterone (MT). In the hybrid sturgeon bester (*Huso huso* female x *Acipenser ruthenus* male), 25 mg/g b.w. diet MT administered to sex-differentiated fish (14–31 months of age) failed to induce masculinization and resulted in approximately 30 % undeveloped gonads (Omoto et al., 2002). Notably, high doses of MT (50 mg/kg b.w. diet) administered to ship sturgeon gynogens (*Acipenser nudiventris*) from 5 to 12 months of age induced complete masculinization; lower doses (10 mg/kg b.w. diet) failed to produce this effect (Saber et al., 2019).

In the Siberian sturgeon, the natural 11-oxigenated androgen (11β-And) administered to genetic females during the undifferentiated gonadal stage was unable to repress the feminine pathway at a dosage of

5 mg/kg b.w. via injection. Some transient signs of masculinization were observed at 6 and 9 months of age, but in no case did a male gonad emerge. At 12 months of age, the gonads of androgen-treated females showed the typical characteristics of an ovary but at varying stages of development. Androgens seem to accelerate the development of primary growth oocytes in some females, a finding that remains to be confirmed in larger numbers of fish.

In previous studies in trout, 11β-And at 10 mg/kg b.w. diet induced an enormous repression of two female genes, *cyp19a1* and *foxl2*, leading to a female-to male sex transdifferentiation, even inducing all stages of spermatogenesis (Vizziano et al., 2008).

In contrast to these observations in trout, the 11β-And treatment applied in the present work did not significantly suppress *cyp19a1* and *foxl2* expression. These two genes have been validated as very early markers of ovarian development in Siberian sturgeon at 3 months of age, when the gonads remain completely undifferentiated (Lasalle et al., 2024; Lasalle et al., 2021; Vizziano-Cantonnet et al., 2018a, 2018b). It is possible that, as has been proposed for several teleosts (Guiguen et al., 2010), masculinization in sturgeon depends more heavily on repression of aromatase than any direct effect of androgens. In our previous work, we observed the androgen receptor expression in fish gonads at 2.5 and 3 months of age (Vizziano-Cantonnet et al., 2018b), and recently, it was stated that *cyp11c* (the key gene that allows for transformation of aromatizable androgens into 11-oxigenated androgens) is not sexually dimorphic in genetically-sexed, undifferentiated male Siberian sturgeon (Lasalle et al., 2024). These results, along with the fact that 11β-And does not have profound effects on the gonads of genetically female individuals in terms of inducing a change in gonadal phenotype, suggest that androgens are not the main drivers of male sex differentiation and instead supports an 11-oxygenated androgen-independent process of natural masculinization of Siberian sturgeon, as proposed previously (Lasalle et al., 2024).

Once again, we cannot compare doses given through feeding versus injection. However, it is interesting to note that low doses of MT (1 mg/g b.w. diet) administered during an undifferentiated stage (3 months of age) over a period of 15 months provoked masculinization of all treated fish, according to testing performed at 9 months (Omoto et al., 2002). Here, the use of 11β-And induced transient signs of disorganization in female gonads, but these changes were not sustained after the treatments were discontinued. In the present work, the androgen selected, at the dosage, treatment duration, and timing applied, failed to induce female-to-male gonadal transdifferentiation as expected, in contrast with the clear feminization effect of estrogens used at the same dosage and timing.

4.3. Conclusion

The experimental feminizing protocols induced complete gonadal transdifferentiation of genetically-sexed males into a phenotypic female. The labile period for sex change in Siberian sturgeon corresponds to an early stage in which the gonads remain undifferentiated. E2 treatment, administered during this undifferentiated period, brought about a process of transdifferentiation mimicking the natural gonadal development of females.

In contrast, 11-oxygenated androgens failed to repress the feminine pathway at the molecular or morphological level at the experimental dosage, treatment duration, and timing. Other hormones or chemicals should be tested to attempt gonadal masculinization.

The experimental conditions produced live, sex-inverted fish with normal growth, feeding, and mobility as compared to controls. Such fish could be crossed with normal fish to further investigate the Siberian sturgeon sex-determination system.

Funding

This work was supported by the Agencia Nacional de Investigación e Innovación Project: FMV_1_2021_1_167027.

CRediT authorship contribution statement

Denise Vizziano-Cantonnet: Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **German Benech-Correa:** Visualization, Validation. **André Lasalle:** Validation, Supervision, Methodology.

Declaration of competing interest

We have no conflicts of interest to declare.

Acknowledgements

We are grateful to the Chief Executive Officer of Estuario del Plata, Facundo Márquez, and the entire Estuario del Plata (Uruguay) staff for their ample support and kindness during this project.

Data availability

No data was used for the research described in the article.

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