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Establishment of intraperitoneal germ cell transplantation for critically endangered Chinese sturgeon *Acipenser sinensis*



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ABSTRACT

Recent progress in germ cell transplantation techniques in fish has paved the way for the conservation of endangered species. Here, we developed an intraperitoneal germ cell transplantation procedure using Chinese and Dabry's sturgeon as donor and recipient species, respectively. Histological analysis revealed that primordial germ cells migrated on the peritoneal wall at 16 days post-hatch (dph) in Dabry's sturgeon. The genital ridges of Dabry's sturgeon (recipient) first formed at 28 dph, suggesting that for successful colonization of donor germ cells in the recipient gonads, the transplantation should be performed earlier than this age. Sexual dimorphism of gonadal structure was first observed at 78 dph. Gonadal germ cell proliferation was not seen in either sex during this period. Immunohistochemistry using the anti-Vasa antibody found that donor testes from 2-year-old Dabry's sturgeon mainly consisted of single- or paired-type A spermatogonia, while donor ovaries from 11.5-year-old Chinese sturgeon had perinucleolus stage oocytes and clusters of oogonia. Donor cells isolated from Dabry's sturgeon testes or Chinese sturgeon ovary labeled with PKH26 fluorescent dye were transplanted into the peritoneal cavity of the 7- or 8-dph Dabry's sturgeon larvae. More than 90% and 70% of transplanted larvae survived after 2 days post-transplantation (dpt) and 51 dpt, respectively. At 51 dpt, PKH26-labeled cells exhibiting germ cell-specific nuclear morphology and diameter were observed in excised recipient gonads by fluorescent and confocal microscopy. The colonization rate of allogeneic testicular germ cell transplantation (Group 1) was 70%, while that of two batches of xenogeneic ovarian germ cell transplantation (Group 2 and Group 3) were 6.7% and 40%, respectively. The ratio of colonized germ cells to endogenous germ cells was 11.96%, 5.35% and 3.56% for Group 1, Group 2 and Group 3, respectively. Thus, we established a germ cell transplantation technique for the critically endangered Chinese sturgeon using the most closely related species as a recipient and demonstrated the successful preparation of transplantable female germ cells from aged adult Chinese sturgeon.

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1. Introduction

¹ These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.theriogenology.2017.02.009 0093-691X/© 2017 Elsevier Inc. All rights reserved. Sturgeons (Chondrostei, Acipenseriformes) represent an ancient Actinopterygian lineage that are called "living fossils" [1]. Three endemic sturgeon species are found in the Yangtze River [2]: Chinese paddlefish (*Psephurus gladius*), Chinese sturgeon (*Acipenser sinensis*) and Dabry's sturgeon (*Acipenser dabryanus*). All are critically endangered or possibly extinct [2–5]. Chinese sturgeon was listed as a Category I nationally protected species in China in 1988 and as a critically endangered species on the International Union

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for Conservation of Nature (IUCN) Red List in 2010 [4].

Chinese sturgeon is a typically large anadromous fish that spends its growth phase in waters off the continental shelf of East Asia and spawns in the upper reaches of rivers [2]. Its numbers in nature have declined dramatically over the past three decades, especially after construction of the Gezhouba Dam [6], and many efforts have been undertaken since 1983 to save this species [2.6] and to even develop it into an aquaculture species due to its fast growth and large egg size, which is an advantage for the market value of caviar. Despite efforts to conserve this species through imposing a fishing ban, constructing nature reserves and rearing fish in captivity for stocking, including with the use of artificial propagation, the situation appears to remain more serious than expected. Our observations of Chinese sturgeon spawning grounds found no evidence of spawning activity during the past three spawning seasons (2013, 2014 and 2015) [6, OW unpublished]. The remaining natural population is on the verge of extinction, but the discovery of four wild juvenile Chinese sturgeon in Yangtze Estuary on April 16, 2015 indicates that a few individuals had spawned at an unknown or even new spawning ground in 2014 [7].

Chinese sturgeon has a long life span (over 30 years), late sexual maturity (8-26 years), and a large body size (an average size of 273 cm in total length and 213.7 kg in body weight) [2]. Previously, we succeeded in producing F1 fish using artificial propagation with wild-caught Chinese sturgeon and then reared those fish for 12-15 years in captivity [8]. In 2012, only two out of 250 F1 females reached the vitellogenic stages (stage III and IV) in our facility, and one successfully spawned viable eggs after hormone treatment [8]. The ovaries of other F1 females still remained at the previtellogenic stage (stage II). To date, no F2 offspring have reached sexual maturity. Thus, in order to increase the likelihood of obtaining eggs through ovulation of Chinese sturgeon in captivity, further investigation of the reproductive characteristics, appropriate nutrition and aquaculture conditions of this fish need to be optimized. When considering the rapid decrease in numbers of this species in the wild and the difficulty of applying conventional artificial reproduction methods, such as hormone treatments and rearing in captivity under a controlled rearing environment due to their large size and late sexual maturation, new methods are needed to facilitate artificial propagation of this species from both aquaculture and conservation viewpoints.

In the past decade, an innovative technique for producing fish surrogate broodstock has been developed in which primordial germ cells (PGCs), spermatogonia, or ogonia from a target fish species (donor) are intraperitoneally transplanted into the larvae of a closely related species (recipient) that is easy to rear to maturity in captivity, resulting in the production of sperm and eggs of the donor in the recipients [9-13]. This technique was first successfully demonstrated in the production of rainbow trout (Oncorhynchus mykiss) from masu salmon (Oncorhynchus masou) recipients. Interestingly, trout spermatogonia and oogonia differentiated into functional eggs in the recipient ovary and sperm in the recipient testis following transplantation into newly hatched salmon, revealing a high level of sexual plasticity of mitotic germ cells [11–13]. Because donor germ cells could be prepared without restriction of donor fish sex, these findings improved the feasibility of germ cell transplantation in fish, especially when working with endangered or rare species. This technique has been expanded across a variety of teleosts, including freshwater fish, such as zebrafish [14] and sturgeon [15], and marine fish, such as Nibe croaker [16-18], mackerel [19,20] and yellowtail [21,22]. In sturgeon, Pšenička et al. [15] demonstrated that donor germ cells isolated from Siberian sturgeon (Acipenser baerii) ovary and testis could be colonized to and proliferate in the gonads of Sterlet (Acipenser ruthenus) larvae, suggesting the promise of applying germ cell transplantation to other sturgeon species.

Intraperitoneally transplanted xenogenic, or even interfamily donor germ cells, could colonize the recipient gonads [17,19,22], indicating that the mechanism of germ cell migration to the embryonic gonads, i.e. genital ridges, is well conserved across fish species. However, progression of xenogenic donor-derived gametogenesis in the recipient gonads might be controlled by other factors, such as cell-cell interactions and molecular signaling. which exist between xenogenic germ cells and gonadal somatic cells [19,22]. Among the intergenus donor-recipient combinations tested to date in the intraperitoneal germ cell transplantation system, successful production of donor-derived sperm was only reported in Carangidae using Japanese yellowtail (Seriola quinqueradiata) and jack mackerel (Trachurus japonicas) as donor and recipient, respectively [20], although production of allogenic or intragenus donor-derived sperm and eggs has been reported in several species, such as salmonids [10,12], zebrafish [14], tilapia [23], Japanese yellowtail [21] and Nibe croaker [16]. At present, however, the mechanisms hindering xenogenic gametogenesis in recipient gonads are not understood, necessitating the selection of a phylogenetically close species as a recipient for Chinese sturgeon.

Dabry's sturgeon is a freshwater fish with a small size (body length, 130 cm; body weight, BW, 16 kg) that is mainly distributed in the main part of the upper Yangtze River and its tributaries [24]. Male and female Darby's sturgeon in nature reach sexual maturity at 4–6 and 6–8 years, respectively [24], and they both reach sexual maturity in a much shorter time in aquaculture at 3 and 5 years, respectively. Though Dabry's sturgeon is classified as being a critically endangered species [5], there are hundreds of broodstock and thousands of adults and juveniles at our institute and the Yibin Institute of Rare Aquatic and Terricolous Animals. More importantly, the life cycle of Dabry's sturgeon from artificial propagation to rearing larvae to maturity has been successfully completed in captivity under fully controlled conditions, producing tens of thousands of Dabry's sturgeon larvae every year. Additionally, among sturgeon, Dabry's sturgeon is most closely related to Chinese sturgeon [25,26]. Since Dabry's sturgeon seems to be the most suitable recipient to support germ cell transplantation of Chinese sturgeon, we aimed to establish an intraperitoneal germ cell transplantation procedure in this study using hatchlings of Dabry's sturgeon as recipients and ovarian and testicular germ cells of Chinese and Dabry's sturgeon, respectively, as donors.

2. Material and methods

2.1. Animal ethics

All fish handling and experimental procedures were approved by the Animal Care and Use Committee of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences.

2.2. Fish

Dabry's sturgeon was reared at Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. In this study, six female and ten male of Dabry's sturgeon were used for artificial propagation in April 2015 and 2016. The broodstock was kept in tanks at 18 ± 1 °C. To induce ovulation and spermiation of Dabry's sturgeon, hormones were injected intramuscularly at the base of the pectoral fin. Males were treated with a single injection of luteinizing hormone-releasing hormone (LHRH) at a dose of 3 µg/kg body weight (BW). At about 12 h after hormone injection, sperm was collected from the urogenital papilla and stored at 4 °C until fertilization. In females, ovulation was induced with two injections (first, 0.4 µg/kg BW LHRH; second, 8 µg/kg BW LHRH and 1 mg/kg BW domperidone, 12 h after the first injection). Eggs were collected at 12–16 h after the second injection. The eggs were fertilized with sperm in aerated groundwater at 19 \pm 1 °C. Stickiness of the fertilized eggs was removed by treating with clay water. After embryos hatched, they were reared in 1600-L tanks with initial stocking density of four individuals per liter. Larvae were fed with the sludge worm *Tubifex* sp. for about 15 days after the yolk digested, followed by gradual feeding of artificial diets. Larvae rearing was carried out at the same temperature and environmental conditions in 2015 and 2016.

Six 2-year-old male Dabry's sturgeon (TL, 74.7 \pm 6.2 cm; BW, 2.2 \pm 0.3 kg; gonad weight, 1.07 \pm 0.89 g; gonadosomatic index, 0.045 \pm 0.032%) were sacrificed, and their gonads were used for donor cell isolation. For Chinese sturgeon, one 11.5-year-old female (TL, 144.3 cm; BW, 15.5 kg; gonad weight, 12.9 g; gonadosomatic index, 0.083%) was used for donor cell isolation.

2.3. Histology

The larvae (TL, <50 mm) and juveniles (TL, >50 mm) hatched from two batches of Dabry's sturgeon eggs obtained in April 2015 were used for histological analysis of early gonadal development. Heads and digestive organs of juveniles were removed by dissection before fixation in Bouin's solution. The testes of Dabry's sturgeon and ovary of Chinese sturgeon used for donor cell preparation were trimmed to 3-mm pieces and fixed in Bouin's solution. Samples were cut into 4-µm thickness in a standard paraffinembedding method and stained with hematoxylin-eosin (HE). Images of sections were obtained using a light microscope (BX-51, Olympus) and a digital camera (DP-73, Olympus).

2.4. Immunohistochemistry

Sections were deparaffinized with xylene, rehydrated through a graded alcohol series, and rinsed in phosphate-buffered saline (PBS). Antigen retrieval was performed by incubating the slides in HistoVT One (Nacalai Tesque, Inc.) for 30 min at 95 °C. Slides were cooled to room temperature and then wash with PBS three times for 5 min, followed by a blocking step using 10% goat serum for 4 h. The slides were washed with PBS once for 5 min and then incubated overnight at 4 °C with Dabry's sturgeon anti-Vasa antibody (1:250) [27]. Slides were washed with PBST five times for 10 min and PBS two times for 10 min. After that, slides were incubated with goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor[®] 488 conjugate (Invitrogen) for 1 h. Incubation solution was removed with PBS, and the slides were washed with PBS 5 times for 10 min. The cell nuclei were stained with 5 µM of 4', 6'-diamidino-2phenylindole (DAPI) for 10 min. and excess stain was removed by washing the slides with PBS 3 times for 10 min and covering the slides with SlowFade[®] Gold antifade reagent (Invitrogen). Images were taken under a fluorescent microscope (BX51-34FL, Olympus) with a digital camera (DP-73, Olympus). The coverslip was removed and the sample was stained with HE and photographed again. Spermatogonia and oogonia were randomly selected, and their nucleus diameter (n = 30) were measured. The results were reported as mean \pm standard error of mean (SEM).

For whole-mount immunohistochemistry of gonad, digestive organs and head of larvae were removed by dissection, and the rest of the body was fixed with Tissue-Tek Ufix for 1 min on ice. Gonads were further removed by fine forceps and put in wells on a 24-well plate with PBST. The same process as for sections was carried out, except that PBST was used instead of PBS. Samples were visualized under a confocal microscope (Olympus FV1000) and images were recorded.

2.5. Preparation of donor cells

Donor cells were prepared from testes of Dabry's sturgeon and ovary of Chinese sturgeon. Freshly isolated gonads were dissociated according to the method described by Takeuchi et al. [16]. Briefly, gonads were minced, and washed three times in Leibovitz's L-15 medium (Gibco). The washed minced tissue was then incubated with 0.25% trypsin (Worthington), 4 mg/ml collagenase H (Roche), 5% fetal bovine serum (FBS) and 0.05% DNase I (Roche) in L-15 medium. The enzymatic digestion was conducted for 2.5 and 3.5 h for ovary and testes, respectively, at 25 °C. During incubation, gentle pipetting was applied to physically disperse any remaining intact portions of the gonads every 30 min. The resultant cell suspension was sequentially filtered through 150- and 50-µm filters (Sysmex-Partec) to remove non-dissociated cell clumps. To trace donor cells in the recipients after transplantation, donor cells were stained with fluorescent membrane dye PKH26 (Sigma). Approximately 10 million cells were suspended in a solution comprising 0.4 ml of diluent C and 4 µl of PKH26. Diluted dye was incubated with the cells for 5 min at room temperature. The cells were then centrifuged at $200 \times g$ for 5 min at 4 °C, and washed twice with L-15 medium. Finally, about 5.0×10^6 cells were resuspended in 1 ml of L-15 medium with 5% FBS and 0.01% DNase I and stored on ice until use.

2.6. Cell transplantation

Transplantation needles were prepared by pulling glass capillaries (G-100, Narishige) using an electric puller (PC-10, Narishige). The tips of the needles were sharpened with a grinder (EG-400, Narishige) until the opening reached 40-50 µm. Recipient larvae obtained in April 2016 were anesthetized at 7-8 days post-hatch (dph) with 0.001% ethyl 3-aminobenzoic acid ethyl ester methanesulfonate-222 (Sigma). Larvae were transferred to a Petri dish coated with 3% agar using a 10-ml glass pipette. Cell transplantation was performed with a micromanipulator (MN-152, Narishige) and microinjector (IM-9B, Narishige) connected to a dissection microscope (SZX10, Olympus). About 200 µl of prepared cell suspension was placed on the glass slide with a shallow cavity. After cells settled at the bottom of the cavity, the tip of needle was placed below the liquid level of the cell suspension to draw up approximately 50 nl of cell suspension attached to the bottom by the oil pressure of the microinjector. The loaded needle was inserted into the peritoneal cavity of the recipient larva, and approximately 50 nl of donor cell suspension containing 5.0×10^4 cells was injected. After transplantation, recipient larvae were placed in a recovery tank, and then were reared in a 400-L tank until fluorescent microscopy observation.

2.7. Fluorescent observation of donor-derived germ cells in recipient Dabry's sturgeon

To confirm the colonization rate of PKH26-labeled donor cells into the gonad, a subset of recipient fish at 51 days posttransplantation (dpt) were examined under a fluorescent microscope (DM5000B, Leica). The digestive organs and head of recipient and control fish were removed, and the remaining intact tissue was fixed with the Tissue-Tek Ufix for 1 min on ice, followed by two rinses in PBS. Gonads were then carefully removed by fine forceps, stained with DAPI for 10 min, washed three times with PBS, and placed on a glass slide (Matsunami) covered with SlowFade[®] Gold antifade reagent (Invitrogen). Nuclear morphology of PKH26labeled cells incorporated into the recipient gonads was observed with a confocal microscope (FV1000, Olympus).

 Table 1

 Age and size of Dabry's sturgeon used for early gonadal development studies.

Age (dph)	No. of fish	TL (mm, mean \pm SEM)	BW (g, mean \pm SEM)
16	5	21.76 ± 0.20	0.026 ± 0.001
28	5	31.64 ± 0.58	0.102 ± 0.005
36	5	43.86 ± 0.36	0.259 ± 0.008
60	5	117.52 ± 2.40	5.018 ± 0.267
78	8	148.09 ± 2.19	12.669 ± 0.558

Abbreviation: dph, days post-hatch; TL, total length; BW, body weight; SEM, standard error of mean.

2.8. Statistical analysis for the nuclear diameter of germ cells

All data are presented as mean values \pm SEM. One-Sample Kolmogorov-Smirnov test confirmed that samples in each group came from populations with normal distributions of nuclear diameter of donor germ cells and colonized PKH26-labeled cells in the recipient gonads (IBM SPSS statistics for Windows, Version 19.0). Bartlett's test confirmed homogeneity of variance of the mean nuclear diameter of cells between groups (p = 0.82), and data could be analyzed by ANOVA and post-hoc Tukey-Kramer's multiple comparisons test to identify significant differences between group means (GraphPad Prism 5). For all statistical tests, values were considered significantly different at p < 0.05.

3. Results

3.1. Early gonadal development in Dabry's sturgeon

According to previous studies [16,19], recipient somatic cells lose the ability to guide donor germ cells to the genital ridges after endogenous PGCs become completely surrounded by precursor of gonadal somatic cells. Thus, we analyzed the histology of early gonadal development of Dabry's sturgeon from 16 to 78 dph (Table 1) to determine when the genital ridges were formed. At 16 dph, migrating PGCs having large and ovoid nuclei were observed along the dorsal epithelium of the peritoneal cavity between the gut and kidney (Fig. 1A). At 28 dph, genital ridges formed and protruded into the peritoneal cavity, and PGCs were partially accompanied by gonadal somatic cells (Fig. 1B). At 36 dph, genital ridges became remarkably elongated ventrally compared to at the previous stage, and gonadal somatic cells completely enveloped the PGCs (Fig. 1C). At 60 dph, with the growth of genital ridges, small blood vessels and germinal epithelium, a columnar monolayered epithelium, were visible (Fig. 1D). Two types of gonads were distinguishable at 78 dph. Type I (n = 5, Fig. 1E) was characterized by many notches in the germinal epithelium, while type II (n = 3, Fig. 1F) featured smooth germinal epithelium. Although the gonads increased in volume in accordance with body growth (TL, about 150 mm) and dimorphically differentiated by 78 dph, each PGC was still located as a single isolated germ cell in the gonads and no mitotic stage of PGCs was observed in this study (Fig. 1E). Indeed, it was difficult to find PGCs in the transverse section of gonads of 78-dph juveniles (Fig. 1E and F).

3.2. Identification of germ cells in donor gonads

Immunohistochemistry using Dabry's sturgeon anti-Vasa antibody was performed to examine which types of germ cells were present in donor gonads because previously, only type-A spermatogonia in testis and oogonia in ovary were shown to have the capability to migrate and colonize recipient genital ridges [13,28]. The specificity of Dabry's sturgeon anti-Vasa antibody to germline cells in the gonads was confirmed in our previous study [27], and this antibody could also be applied in Chinese sturgeon due to very high sequence identity of the Vasa protein between Dabry's sturgeon and Chinese sturgeon [29]. As shown in Fig. 2, a representative image of the testis of a 2-year-old Dabry's sturgeon only consisted of type-A spermatogonia (Fig. 2A–F). In addition to the single type-A spermatogonia, paired type-A spermatogonia were confirmed by anti-Vasa staining (Fig. 2D-F). In the ovary of the 11.5-year-old female Chinese sturgeon, nests of oogonia were observed in the interstitial area (Fig. 2G-L). Perinuleolus stage oocytes with a maximum diameter of about 250 µm were also present in this ovary. Additionally, type-A spermatogonia and oogonia had larger round or ovoid nuclei than gonadal somatic cells (Fig. 2D, F, J and L).

3.3. Testicular and ovarian cell transplantations

After enzymatic digestion, approximately 5.2×10^6 and 9.5×10^6 cells were obtained from a 6.41 g fragment of testis and a 12.9 g fragment of ovary, respectively (Fig. 3A and C). Subsequently,



Fig. 1. Early gonadal development of Dabry's sturgeon. Transverse sections of gonads at 16 dph (A), 28 dph (B), 36 dph (C), 60 dph (D), 78 dph (female; E) and 78 dph (male; F) stained with HE (black bar, 20 µm). Arrowheads indicate PGCs. The insets in D and E showed a high magnification view of the respective primordial germ cells (white bar, 10 µm). BV, blood vessel; PC, peritoneal cavity; DM, dorsal musculature; GE, germinal epithelium; GR, genital ridge; GT, gastrointestinal tract; K, kidney.



Fig. 2. Histological sections of donor gonads. Sections of Dabry's sturgeon testis are stained with HE (A), anti-Vasa antibody (B) and anti-Vasa antibody and DAPI (C). Higher magnification views of the boxed area in A, B and C are shown in D, E and F, respectively. Sections of Chinese sturgeon ovary are shown stained with HE (G), anti-Vasa antibody (H) and anti-Vasa antibody and DAPI (I), and higher magnification views of the boxed area in G, H and I are shown in J, K and L, respectively. A SG, type A spermatogonia; paired A SG, paired type A spermatogonia; OG, oogonia. Bars, 20 μm (A-C and G-I); 10 μm (D-F and J-L).

isolated cells were stained with fluorescent membrane dye, PKH26 (Fig. 3B and D), making it possible to conduct transient tracing of these cells in the recipient's body cavity. The tip of the transplantation needle was injected into the peritoneal cavity of 7- and 8-dph larvae near the posterior intestine (Fig. 3E and F) because we previously demonstrated that endogenous Dabry's sturgeon PGCs are located there [27]. Confirmation of the injection under fluorescence microscopy showed that PKH26-labeled donor cells were present in the peritoneal cavity in the vicinity of the injection (Fig. 3G and I). No fluorescence was observed in non-transplanted larvae (Fig. 3H and J). The survival rate at 48 h posttransplantation was 91.8% (n = 183), and it remained high at 51 dpt (70.5%; Table 2).

3.4. Morphological characteristics of genital ridges and endogenous primordial germ cells in 58 dph fish

Since the precise location of gonads in the body cavity of juvenile Dabry's sturgeon has not been examined, non-transplanted fish at 58 dph (TL, 105.5 \pm 3.5 mm; BW, 3.6 \pm 0.3 g), which corresponds to 51 dpt, were dissected to remove digestive organs and photographed after fixing with Bouin's solution (Fig. 4A). Then, transverse (Fig. 4B and C) and sagittal (Fig. 4D and E) sections of the body were stained with HE, revealing that gonads harboring PGCs with large and round nuclei and abundant cytoplasm were bilaterally attached to the dorsal body wall (Fig. 4A–C). Counts of Vasapositive cells in pairs of whole-mount gonads of non-transplanted fish at 58 dph showed 56.1 \pm 2.25 (n = 8) endogenous PGCs per individual at this stage (Fig. 4F).

3.5. Incorporation of transplanted germ cells into recipient gonads

To examine whether donor-derived germ cells were colonized into the genital ridges of recipients, the presence of PKH26-labeled cells in recipient gonads was investigated at 51 dpt by fluorescence microscopy. As shown in Fig. 5, both allogenic and xenogenic PKH26-labeled cells were found in the gonads of transplanted fish (Fig. 5A and B, donor cells from Dabry's sturgeon testis; Fig. 5C and D, donor cells from Chinese sturgeon ovary), while no fluorescence was detected in the gonads of non-transplanted Dabry's sturgeon (Fig. 5E and F). These data strongly indicated that donor cells were successfully incorporated into the gonads of recipient Dabry's sturgeon and survived to at least 51 dpt.

Since PGCs, type-A spermatogonia and oogonia had larger round or ovoid nuclei than gonadal somatic cells (see Figs. 2D, J and E), recipient gonads harboring PKH26-labeled cells were therefore Table 3



Fig. 3. Intraperitoneal transplantation of donor cells into Dabry's sturgeon larvae. Dissociated cells from Dabry's sturgeon testis in the bright field view (A) and labeled with red fluorescent dye, PKH26 (B). Dissociated cells from Chinese sturgeon ovary in the bright field view (C) and labeled with red fluorescent dye, PKH26 (D). Intraperitoneal transplantation of PKH26-labeled cells into the 7 dph recipient larvae (E) with high magnification of the boxed area shown in (F). Bright field views of transplanted (G) and non-transplanted (H) larvae just after transplantation. Arrowhead indicates transplanted donor cells labeled by PKH26. Bars, 10 μm (A–D); 100 μm (G–J).

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Survival and	colonization	rates of ge	rm cell t	ransplantation	ı in	sturgeon

Group ^a	No. transplanted	Survival rate (%) ^b	No. colonized and observed ^c	Colonization rate (%)	No. colonized cells ^d	No. endogenous germ cells ^e	Ratio of colonized germ cells to endogenous germ cells (%) ^f
Group 1	43	33 (76.7)	7/10	70	6.7 ± 1.34	_	11.96 ± 2.39
Group 2	65	47 (72.3)	1/15	6.7	3	-	5.35
Group 3	75	49 (65.3)	4/10	40	2 ± 0.71	-	3.56 ± 1.26
Control	58	50 (86.2)	0/10	0	0	56.1 ± 2.25	0

^a Group 1, cells isolated from Dabry's sturgeon testis were injected into the larvae of Dabry's sturgeon at 8 days post-hatch. Group 2 and 3, cells isolated from Chinese sturgeon ovary were injected into the larvae of Dabry's sturgeon at 7 and 8 days post-hatch, respectively. Control, non-transplanted Dabry's sturgeon larvae.

^b Based on number of viable recipients at 51 days post-transplantation.

^c Number of recipients colonized by donor-derived germ cells at about 51 days post-transplantation, and the total number of recipients for which observations were made.

^d Values are mean ± standard error of mean.

^e Number of endogenous germ cells of Dabry's sturgeon at 58 days post-hatch obtained by immunohistochemistry with anti-Vasa antibody reported as mean \pm SEM. ^f Ratio of colonized germ cells to endogenous germ cells (%) = number of colonized germ cells/average number of endogenous germ cells at 58 days post-hatch \times 100.

Values are shown as mean \pm standard error of mean.

stained with DAPI and observed under a confocal microscope to determine whether colonized PKH26-labeled cells (Fig. 6A and D) had germ cell-specific nuclei. In allogenic testicular cell transplantation, cells with large round nuclei (red and yellow arrowheads in Fig. 6B) were distinguished from somatic cells with nonround nuclei by DAPI staining. The merged image of DAPI and PKH26 signals confirmed that some of the cells with large round nuclei (red arrowheads in Fig. 6B and C) were labeled by PKH26, suggesting that donor-derived spermatogonia had colonized in the recipient gonad. In contrast, cells indicated by yellow arrowheads in Fig. 6B were most likely endogenous PGCs. In xenogenic Chinese sturgeon ovarian cell transplantation, cells having germ cellspecific characters (red arrowheads in Fig. 6D-F) were positive for PKH26, suggesting that ovarian germ cells prepared from 11.5year-old Chinese sturgeon colonized Dabry's sturgeon gonads. In non-transplanted fish, no PKH26-positive cells were found (Fig. 6G–I), although endogenous PGCs with large round nuclei (yellow arrowheads in Fig. 6H and I) were observed. These results indicate that the incorporated PKH26-labeled cells in the recipient gonads were donor-derived germ cells.

In order to further demonstrate whether colonized PKH26labeled cells were donor-derived germ cells, the nucleus diameter of recipient PGCs, donor germ cells, and colonized PKH26-labeled cells were compared. As shown in Fig. 7, the nucleus diameter of Chinese sturgeon oogonia (OG, $6.41 \pm 0.27 \ \mu\text{m}$, n = 30) was significantly smaller than those of Dabry's sturgeon PGCs ($8.13 \pm 0.13 \ \mu\text{m}$, n = 30) and spermatogonia (SG, $7.82 \pm 0.23 \ \mu\text{m}$, n = 30) (p < 0.05). Notably, the nucleus diameter of colonized PKH26-labeled testicular (n = 12) and ovarian (n = 5) cells was $7.81 \pm 0.21 \ \mu\text{m}$ and $6.86 \pm 0.28 \ \mu\text{m}$, respectively, which were not significantly different from those of spermatogonia (SG) and oogonia (OG), respectively, (p > 0.05). Thus, consistent patterns in nucleus diameter of colonized PKH26-labeled cells and germ cell donor cells provided further evidence that these cells are donor-derived germ cells.

The colonization rate of colonized donor cells to endogenous PGCs determined at 51 dpt (Table 2) was 70%, 6.7% and 40% in the gonads of Group 1 (n = 10), Group 2 (n = 15) and Group 3 (n = 10), respectively, with a mean number of donor-derived germ cells of 6.7 \pm 1.34 (n = 7), 3 (n = 1) and 2 \pm 0.71 (n = 4), respectively. Additionally, the ratio of colonized germ cells to endogenous PGCs (= number of colonized PKH26-positive germ cells/average number of endogenous germ cells at 58 dph \times 100) was 11.96 \pm 2.39%, 5.35% and 3.56 \pm 1.26% for Group 1 (n = 7), Group 2 (n = 1) and



Fig. 4. Morphological characterization and number of primordial germ cells in gonads of non-transplanted recipients at 58 dph. Non-transplanted recipient removed digestive organs and head are photographed after fixing with Bouin's solution (A). Transverse (B) and sagittal (D) sections of non-transplanted recipient gonad stained with HE and areas enclosed in boxes are shown in (C) and (E), respectively. The inset in C is a high magnification view of a primordial germ cell (Bar, 10 µm). Whole-mounts immunohistochemistry of gonads from a non-transplanted recipient were prepared to determine the number of primordial germ cells. The gonads were stained by the anti-Vasa antibody (F) and are shown in the bright field view (G). Primordial germ cells are indicated by arrowheads. Bars, 10 µm (E); 20 µm (C and D); 100 µm (A, F and G); 200 µm (B).



Fig. 5. Incorporation of transplanted germ cells from Dabry's sturgeon testis-derived cells (A, B), Chinese sturgeon ovary-derived cells (C, D) into Dabry's sturgeon recipient genital ridges and a non-transplanted Dabry's sturgeon (E, F) at 51 dpt. View under bright field are shown in A, C and E, and under fluorescence microscopy are shown in B, D and F. Arrowheads indicate colonization of donor-derived PKH26-labeled cells to the recipient gonad. Bars, 20 μm.



Fig. 6. Morphological characterization of excised gonads from Dabry's sturgeon testis-derived cells (A, B and C), Chinese sturgeon ovary-derived cells (D, E and F) into Dabry's sturgeon recipient and a non-transplanted Dabry's sturgeon (G, H and I) at 51 dpt. PKH26-labeled cells colonizing recipient gonads observed by confocal microscopy (A, D), and DAPI-stained cells (B, E) and merged image of PKH26 and DAPI staining (C, F). Recipient-derived and donor-derived germ cells are indicated by yellow and red arrowheads, respectively. Bars, 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Fig. 7. Nucleus diameter of Dabry's sturgeon primordial germ cells (PGC), Dabry's sturgeon spermotogonia (SG), Chinese sturgeon oogonia (OG), and colonized PKH26-labeled Dabry's sturgeon testicular cells (colonized cells in SGTP) and colonized PKH26-labeled Chinese sturgeon ovarian cells (colonized cell in OGTP). Means with different letters are statistically different (Tukey's test, P < 0.05).

Group 3 (n = 4), respectively.

4. Discussion

The present study demonstrated the success of intraperitoneal germ cell transplantation for Chinese sturgeon using newly hatched Dabry's sturgeon larvae as recipients. For the first time, we describe the timing of genital ridge formation and dimorphic gonadal differentiation of Dabry's sturgeon, facilitating the selection of a suitable stage for use as recipients of donor germ cells. Confocal analysis of recipient gonads at 51 dpt revealed that the morphological character and nuclear diameter of colonized PKH26labeled cells were reflective of those of donor spermatogonia and oogonia, suggesting that both allogenic and xenogenic donor germ cells were incorporated in the recipient gonads. These results indicate that the somatic microenvironment of the genital ridges of Dabry's sturgeon support survival and colonization of xenogeneic Chinese sturgeon oogonia as well as allogeneic Dabry's sturgeon spermatogonia. This is a first step toward the production of critically endangered Chinese sturgeon gametes using the closely related Dabry's sturgeon as surrogate broodstock.

Due to the limited number and generations of Chinese sturgeon adults, we cannot be selective with respect to the sex and age of fish used as the source of donor germ cells. Among the artificially produced Chinese sturgeon that are more than 10 years old in our facility, which are nearly the only individuals of this species that can be sacrificed for research and used as potential sources of their genetic material for conservation of the species, most of females possess ovaries at pre-vitellogenic stages, while some of the males produced sperm. In rainbow trout, Yoshizaki et al. [13] reported that only oogonia among the dissociated ovarian cells could repopulate the recipient genital ridges. More importantly, the colonized rainbow trout oogonia possessed high self-renewal activity (i.e., stemness) and sexual plasticity in the recipient gonads. Thus, the most remarkable achievement of this study was that oogonia isolated from an 11.5-year-old Chinese sturgeon successfully colonized the genital ridges of recipients. In the future, transplantable oogonia prepared from this adult Chinese sturgeon may proliferate and differentiate as the gametogenesis proceeded in the recipient Dabry's sturgeon. Several studies have suggested that the sexdetermination system of sturgeons is ZZ-male/ZW-female [30,31]. If so, only Z eggs would be obtained when spermatogonia (ZZ) are transplanted into female recipients, resulting in all-male progeny. However, transplanting oogonia (ZW) allows the production of both Z and W eggs in female recipients, leading to the production of both male (ZZ) and female (ZW) F1 offspring when crossed with normal ZZ males. Thus, oogonia transplantation will be required to produce females in the F1 population for conservation purposes, and the method to do so has been established in this research.

Generally, colonization rates obtained in oogonial transplantations are lower than those of spermatogonial transallogeneic plantations. For example, in spermatogonia transplantations, a colonization rate of 85.7% was reported in yellowtail [21] and that of 36.3% was obtained in Nibe croaker [16]. High colonization rate was also reached in xenogenic spermatogonia transplantations, such as chub mackerel transplanted with Nibe croaker testicular germ cells (70%) [19], Nibe croaker transplanted with yellowtail testicular germ cells (81.8%) [17], jack mackerel transplanted with yellowtail testicular germ cells (88.2%) [20], and sterlet transplanted with Siberian sturgeon testicular germ cells (60% at 50 dpt) [15]. In oogonial transplantations, 41.3% of triploid sterile salmon recipients had trout ovarian germ cells at 20 dpt [13], and 20% of sterile Danio hybrid recipients carried donor-derived ovarian germ cells at 42 dpt [14]. In the present study, the colonization rate of allogeneic donor spermatogonia (Group 1 (70%)) was higher than that of xenogenic donor oogonia (Group 2 (6.7%) and Group 3 (40%)) at 51 dpt. Although oogonia were prepared from a different species in the present study, the differences in colonization rates were possibly ascribable to the differences in percentages of transplantable germ cells from testis or ovary. Recently, Pšenička et al. [15] prepared donor ovarian germ cells by percoll gradient centrifugation, giving rise to 70% of sterlet recipients possessing donor-derived Siberian sturgeon ovarian germ cells at 50 dpt. Thus, further enrichment of transplantable oogonia, such as through cell sorting by percoll gradient centrifugation or FACS [32] after the normal enzymatic dissociation of Chinese sturgeon ovary may increase the colonization rates of ovarian germ cells. From another point of view, as reported by Shikina et al. [33], short-term in vitro culturing could allow donor germ cells "damaged" by protease treatment to recover the surface proteins required for efficient incorporation into the recipient genital ridge. The transplantation assay method developed in this study will be useful for evaluating colonization rates of donor germ cells prepared by different protocols.

Previous reports in salmonids [9] and marine perciforms [16,21,23] demonstrated that there was a very narrow window in the developmental stage of recipient larvae during which donor germ cells could colonize into the gonads. Colonization rates would be dramatically decreased, even to zero, if the endogenous PGCs of the genital ridges in the recipient larvae had already been enclosed and were protruding into the body cavity. Interestingly, the colonization rate was lower when donor germ cells were transplanted into the body cavity of younger recipients with endogenous PGCs in the early migration phase and for which somatic counterparts had not formed the gonadal anlage, suggesting that immediate contact with the gonadal anlage to establish the germ-somatic cell interactions is necessary for survival and/or efficient colonization in recipient gonads [19]. According to the histology of Dabry's sturgeon gonadal development at 16 dph, endogenous PGCs were still in the early migration phase, and their genital anlage had not yet been formed. Thus, judging only from the morphological gonadal development, the suitable stage for obtaining efficient colonization of donor germ cells during transplantation may be between 16 and 28 dph. However, formation of genital ridges in Dabry's sturgeon was very slow and occurred at 4 weeks after the hatching stage, producing the situation of the immune system becoming established before the microenvironment of the recipient body cavity had become suitable for accepting donor germ cells. In this study, donor cells transplanted into the body cavity of larvae at 7–8 dph were incorporated into the recipient genital ridges, suggesting that the immune system was not yet fully established at this age. If the development of the immune system of Dabry's sturgeon, which is mostly correlated to development of lymphoid organs such as kidney, spleen and thymus [34], was still immature at above mentioned stages (16-28 dph), it could be possible to improve the colonization rates by delaying the age of recipients at transplantation.

In Dabry's sturgeon, two types of gonads were observed at 78 dph: one had a germinal epithelium with notches while another had a flat germinal epithelium. It was previously reported in other sturgeon species that the former type was presumed to be ovaries, and the latter type was putative testes [35-37], suggesting that gonadal sex differentiation of Dabry's sturgeon was initiated at this stage. The timing of gonadal sex differentiation seems to be species-specific, although timing is possibly influenced by environmental factors [38]. Gonadal sex differentiation occurred at 3 months in Russian sturgeon [39], at 4 months in Siberian sturgeon [37], at 6 months in bester [40], shortnose sturgeon [36], Adriatic sturgeon [35] and Amur sturgeon [41], at 8 months in sterlet [42], and at 9 months in Chinese sturgeon [43]. Thus, the phenotypic sex of Dabry's sturgeon gonads can be identified earlier than in other sturgeon species. Due to the high commercial value of sturgeon eggs (i.e., caviar), monosex all-female seed is desired in aquaculture. Treatment with sex steroids is a widely used technique for achieving monosex production in fish aquaculture [44]. Thus, the morphological information of the timing of sex differentiation is essential for inducing feminized Dabry's sturgeon by estradiol.

The transplanted Dabry's sturgeon are now being reared in our facility, they are expected to reach sexual maturity in 3–5 years. In our previous intra-genus transplantation study between rainbow trout (donor) and diploid masu salmon (recipient) [10], the frequency of recipients that produced donor-derived gametes (i.e., surrogate parent rate) at 1-year-old (13.5%) was not substantially lower than the rate of genital ridge colonization by donor-derived germ cells (i.e., colonization rate) observed at 30 dpt (16.7%). Thus, we expected that Dabry's sturgeon recipient would produce

intra-genus Chinese sturgeon donor-derived gametes in the future. Meanwhile, future germ cell transplantation work using Chinese sturgeon donors will focus on 1) using sterile Dabry's sturgeon recipients produced by knockdown of genes required for germ cell development [45] to obtain a high rate of donor-derived gametes from sexually mature recipients, 2) cryopreservation of donor germ cells or gonads [46,47] to increase the number of replicates of transplantations for each donor individual, and 3) excision of a piece of gonadal tissues by surgery to harvest donor cells without killing endangered Chinese sturgeons.

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References

- Bemis WE, Findeis EK, Grande L. An overview of Acipenseriformes. Sturgeon biodiversity and conservation: Springer. 1997. p. 25–71.
- [2] Wei Q, Fe Ke, Zhang J, Zhuang P, Luo J, Zhou R, et al. Biology, fisheries, and conservation of sturgeons and paddlefish in China. Env Biol Fish 1997;48: 241–55.
- [3] Zhang H, Wei Q, Du H, Shen L, Li Y, Zhao Y. Is there evidence that the Chinese paddlefish (*Psephurus gladius*) still survives in the upper Yangtze River? Concerns inferred from hydroacoustic and capture surveys, 2006–2008. J Appl Ichthyol 2009;25:95–9.
- [4] Qiwei W. Acipenser sinensis. The IUCN red list of threatened species. 2010. e.T236A13044272.
- [5] Zhang H, Wei Q, Du H, Li L. Present status and risk for extinction of the Dabry's sturgeon (*Acipenser dabryanus*) in the Yangtze River watershed: a concern for intensified rehabilitation needs. | Appl Ichthyol 2011;27:181–5.
- [6] Wu J, Wang C, Zhang H, Du H, Liu Z, Shen L, et al. Drastic decline in spawning activity of Chinese sturgeon *Acipenser sinensis* Gray 1835 in the remaining spawning ground of the Yangtze River since the construction of hydrodams. J Appl Ichthyol 2015;31:839–42.
- [7] Zhuang P, Zhao F, Zhang T, Chen Y, Liu J, Zhang L, et al. New evidence may support the persistence and adaptability of the near-extinct Chinese sturgeon. Biol Conserv 2016;193:66–9.
- [8] Wei Q, Li L, Du H, Zhang X, Xiong W, Zhang H, et al. Research on technology for controlled propagation of cultured Chinese sturgeon (*Acipenser sinensis*). J Fish Sci China 2013;20:1–11 [In Chinese with English abstract].
- [9] Takeuchi Y, Yoshizaki G, Takeuchi T. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol Reprod 2003;69:1142–9.
- [10] Takeuchi Y, Yoshizaki G, Takeuchi T. Biotechnology: surrogate broodstock produces salmonids. Nature 2004;430:629–30.
- [11] Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T, Yoshizaki G. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc Natl Acad Sci U. S. A 2006;103:2725–9.
- [12] Okutsu T, Shikina S, Kanno M, Takeuchi Y, Yoshizaki G. Production of trout offspring from triploid salmon parents. Science 2007;317:1517.
- [13] Yoshizaki G, Ichikawa M, Hayashi M, Iwasaki Y, Miwa M, Shikina S, et al. Sexual plasticity of ovarian germ cells in rainbow trout. Development 2010;137:1227–30.
- [14] Wong TT, Saito T, Crodian J, Collodi P. Zebrafish germline chimeras produced by transplantation of ovarian germ cells into sterile host larvae. Biol Reprod 2011;84:1190-7.
- [15] Pšenička M, Saito T, Linhartová Z, Gazo I. Isolation and transplantation of sturgeon early-stage germ cells. Theriogenology 2015;83:1085–92.
- [16] Takeuchi Y, Higuchi K, Yatabe T, Miwa M, Yoshizaki G. Development of spermatogonial cell transplantation in Nibe croaker, *Nibea mitsukurii* (Perciformes, Sciaenidae). Biol Reprod 2009;81:1055–63.
- [17] Higuchi K, Takeuchi Y, Miwa M, Yamamoto Y, Tsunemoto K, Yoshizaki G. Colonization, proliferation, and survival of intraperitoneally transplanted yellowtail *Seriola quinqueradiata* spermatogonia in nibe croaker *Nibea mitsukurii* recipient. Fish Sci 2011;77:69–77.
- [18] Yoshikawa H, Takeuchi Y, Ino Y, Wang J, Iwata G, Kabeya N, et al. Efficient production of donor-derived gametes from triploid recipients following intra-peritoneal germ cell transplantation into a marine teleost, Nibe croaker (*Nibea mitsukurii*). Aquaculture 2016. http://dx.doi.org/10.1016/ j.aquaculture.2016.05.011.
- [19] Yazawa R, Takeuchi Y, Higuchi K, Yatabe T, Kabeya N, Yoshizaki G. Chub mackerel gonads support colonization, survival, and proliferation of

intraperitoneally transplanted xenogenic germ cells. Biol Reprod 2010;82: 896–904.

- [20] Morita T, Morishima K, Miwa M, Kumakura N, Kudo S, Ichida K, et al. Functional sperm of the yellowtail (*Seriola quinqueradiata*) were produced in the small-bodied surrogate, jack mackerel (*Trachurus japonicus*). Mar Biotechnol 2015;17:644–54.
- [21] Morita T, Kumakura N, Morishima K, Mitsuboshi T, Ishida M, Hara T, et al. Production of donor-derived offspring by allogeneic transplantation of spermatogonia in the yellowtail (*Seriola quinqueradiata*). Biol Reprod 2012;86: 1–11.
- [22] Bar I, Smith A, Bubner E, Yoshizaki G, Takeuchi Y, Yazawa R, et al. Assessment of yellowtail kingfish (*Seriola lalandi*) as a surrogate host for the production of southern bluefin tuna (*Thunnus maccoyii*) seed via spermatogonial germ cell transplantation. Reprod Fert Dev 2015. http://dx.doi.org/10.1071/RD15136.
- [23] Farlora R, Hattori-Ihara S, Takeuchi Y, Hayashi M, Octavera A, Yoshizaki G, Intraperitoneal germ cell transplantation in the Nile tilapia *Oreochromis* niloticus. Mar Biotechnol 2014;16:309–20.
- [24] Zhuang P, Fe Ke, Wei Q, He X, Cen Y. Biology and life history of Dabry's sturgeon, Acipenser dabryanus, in the Yangtze River. Sturgeon biodiversity and conservation: Springer. 1997. p. 257–64.
- [25] Zhang S, Zhang Y, Zheng X, Chen Y, Deng H, Wang D, et al. Molecular phylogenetic systematics of twelve species of Acipenseriformes based on mtDNAND4L-ND4 gene sequence analysis. Sci China-Life Sci 2000;43:129–37.
- [26] Krieger J, Hett A, Fuerst P, Artyukhin E, Ludwig A. The molecular phylogeny of the order Acipenseriformes revisited. J Appl Ichthyol 2008;24:36–45.
 [27] Ye H, Yue H-M, Yang X-G, Li C-J, Wei Q-W. Identification and sexually
- [27] Ye H, Yue H-M, Yang X-G, Li C-J, Wei Q-W. Identification and sexuality dimorphic expression of vasa isoforms in Dabry's sturgeon (*Acipenser dab-ryanus*), and functional analysis of vasa 3'-untranslated region. Cell Tissue Res 2016;366:203–18.
- [28] Yano A, Suzuki K, Yoshizaki G. Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout vasa regulatory regions. Biol Reprod 2008;78: 151–8.
- [29] Yue H, Li C, Du H, Zhang S, Wei Q, Sequencing and De Novo assembly of the gonadal transcriptome of the endangered Chinese sturgeon (*Acipenser sinensis*). PloS One 2015;10:e0127332.
- [30] Omoto N, Maebayashi M, Adachi S, Arai K, Yamauchi K. Sex ratios of triploids and gynogenetic diploids induced in the hybrid sturgeon, the bester (*Huso* huso female × Acipenser ruthenus male). Aquaculture 2005;245:39–47.
- [31] Flynn S, Matsuoka M, Reith M, Martin-Robichaud D, Benfey T. Gynogenesis and sex determination in shortnose sturgeon, *Acipenser brevirostrum* Lesuere. Aquaculture 2006;253:721–7.
- [32] Hayashi M, Sato M, Nagasaka Y, Sadaie S, Kobayashi S, Yoshizaki G. Enrichment of spermatogonial stem cells using side population in teleost. Biol Reprod 2014;91:1–8.
- [33] Shikina S, Nagasawa K, Hayashi M, Furuya M, Iwasaki Y, Yoshizaki G. Shortterm in vitro culturing improves transplantability of type A spermatogonia in rainbow trout (*Oncorhynchus mykiss*). Mol Reprod Dev 2013;80:763–73.
- [34] Uribe C, Folch H, Enriquez R, Moran G. Innate and adaptive immunity in teleost fish: a review. Vet Med-Czech 2011;56:486–503.
- [35] Grandi G, Giovannini S, Chicca M. Gonadogenesis in early developmental stages of *Acipenser naccarii* and influence of estrogen immersion on feminization. J Appl Ichthyol 2007;23:3–8.
- [36] Flynn S, Benfey T. Sex differentiation and aspects of gametogenesis in shortnose sturgeon Acipenser brevirostrum Lesueur. J Fish Biol 2007;70:1027–44.
- [37] Rzepkowska M, Ostaszewska T. Proliferating cell nuclear antigen and Vasa protein expression during gonadal development and sexual differentiation in cultured Siberian (*Acipenser baerii* Brandt, 1869) and Russian (*Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833) sturgeon. Rev Aquacult 2014;6: 75–88.
- [38] Strüssmann CA, Nakamura M. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol Biochem 2002;26:13–29.
- [39] Akhundov M, Fedorov KY. Early gametogenesis and gonadogenesis in sturgeons, part 1: on criteria for comparative assessment of juvenile gonadal development in the example of the Russian sturgeon, *Acipenser gueldenstaedtii*. J Ichthyol 1991;31:101–14.
- [40] Omoto N, Maebayashi M, Mitsuhashi E, Yoshitomi K, Adachi S, Yamauchi K. Histological observations of gonadal sex differentiation in the F2 hybrid sturgeon, the bester. Fish Sci 2001;67:1104–10.
- [41] Zhang Y, Sun H, Liu X, Qu Q, Sun D. Histological observation of gonadal differentiation and effect of rearing temperature on sex differentiation in Amur sturgeon Acipenser schrenckii. J Fish Sci China 2012;19:1008–17 [In Chinese with English abstract].
- [42] Wrobel K-H, Hees I, Schimmel M, Stauber E. The genus Acipenser as a model system for vertebrate urogenital development: nephrostomial tubules and their significance for the origin of the gonad. Anat Embryol 2002;205:67–80.
- [43] Chen X, Wei Q, Yang D, Zhu Y, Liu Y. Histological studies on gonadal origin and differentiation of cultured *Acipenser sinensis*. J Fish China 2004;28:633–9 [In Chinese with English abstract].
- [44] Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences.

- Aquaculture 2002;208:191–364.
 [45] Linhartová Z, Saito T, Kašpar V, Rodina M, Prášková E, Hagihara S, et al. Sterilization of sterlet Acipenser ruthenus by using knockdown agent, antisense morpholino oligonucleotide, against *dead end* gene. Theriogenology 2015;84:1246-55.
- [46] Kobayashi T, Takeuchi Y, Takeuchi T, Yoshizaki G. Generation of viable fish from cryopreserved primordial germ cells. Mol Reprod Dev 2007;74:207–13.
 [47] Lee S, Iwasaki Y, Shikina S, Yoshizaki G. Generation of functional eggs and sperm from cryopreserved whole testes. Proc Natl Acad Sci U. S. A 2013;110: 1640-5.