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Sterlet (*Acipenser ruthenus*) Surrogate maybe Reduce the Caviar Development of Critically Endangered Beluga (*Huso huso*)

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Abstract:

The preservation and recovery of endangered fish species can be advanced through germ stem cell (GSC) technologies. In this study, we established an intraperitoneal spermatogonial transplantation method using Beluga (*Huso huso*) as the donor and Sterlet (*Acipenser ruthenus*) as the recipient species. This approach aims to generate germline chimeras that could act as surrogates for Beluga reproduction and potentially shorten the time required for caviar production. Beluga testicular cells were dissociated, purified using a discontinuous Percoll gradient, and verified with the germ cell-specific marker Vasa (DDX4). Approximately 58–64% of the isolated cells were Vasa-positive, confirming enrichment. Freshly isolated cells were labeled with PKH26 fluorescent dye and intraperitoneally transplanted into sterilized Sterlet larvae (5–6 days post-hatch), prepared either by antisense morpholino knockdown of the *dead end* (*dnd*-MO) gene or by heat-shock-induced triploidy. Donor cells were tracked at 15, 30, and 60 days post-transplantation: they initially dispersed randomly, but later proliferated and localized within the developing genital ridges of the hosts. Between 30 and 60 days, donor cell numbers increased significantly in both recipient groups ($P < 0.05$). PCR and RT-PCR analyses confirmed the presence of Beluga-derived cells in Sterlet gonads. At 60 days post-transplantation, molecular analyses together with cell counts revealed that *dnd*-MO-treated Sterlet recipients exhibited significantly higher proliferation and retention of Beluga spermatogonial cells than triploid (3n) recipients ($P < 0.05$). These findings provide the first evidence that the Sterlet gonadal somatic microenvironment can support the survival and expansion of Beluga male germ cells. Therefore, Beluga-to-Sterlet spermatogonial transplantation offers a promising strategy for sturgeon conservation and aquaculture.

Key words: Sturgeon conservation, Germ stem cells, Spermatogonial transplantation, Beluga (*Huso huso*), Sterlet (*Acipenser ruthenus*), *dnd*-MO, Aquaculture

introduction:

Sturgeons (family *Acipenseridae*) are among the oldest groups of fish, with evolutionary roots tracing back to the Triassic period. Despite their long history and ecological success, sturgeon

populations have declined drastically in recent decades due to human activities such as overfishing, habitat destruction, river damming, and environmental pollution (Falahatkar et al., 2011; Pšenička et al., 2015). Currently, the majority of sturgeon species are classified as threatened on the International Union for Conservation of Nature (IUCN) Red List, with several species designated as critically endangered. (Pšenička et al., 2015). Although artificial propagation has long been used to maintain sturgeon populations, their distinctive biological traits including large body size, long reproductive cycles, and irregular spawning present considerable challenges to breeding and rearing in captivity. Furthermore, while sperm cryopreservation offers a partial solution, it only preserves male genetic material, leading to the loss of valuable maternal lineages (Psenicka and Saito, 2020). Therefore, the development of effective conservation strategies for sturgeon has become an urgent priority. Germ stem cells (GSCs) comprising primordial germ cells (PGCs), spermatogonial stem cells (SSCs), and early stage oogonial stem cells (OSCs) possess the ability to differentiate into functional gametes of either sex (Lacerda et al., 2014; Pšenička & Saito, 2020). The remarkable differentiation potential of these cells enables their application in long-term preservation as well as in the production of germline chimeras which can transmit the donor's genetic material through a surrogate host (Robles et al., 2016). Studies investigating primordial germ cell (PGC) development and transplantation in sturgeon have laid a critical foundation for the advancement of such biotechnological approaches (Saito et al., 2014; Pšenička et al., 2015).

The use of germline chimera technology has recently been explored as a strategy for the restoration and preservation of endangered species. In this approach, germline stem cells (GSCs) are isolated from the donor species and transplanted into a phylogenetically related host, where they migrate to the genital ridge, proliferate, and differentiate within the recipient's gonads (Yoshizaki & Yazawa, 2019). This approach was first successfully demonstrated in salmonids, where rainbow trout (*Oncorhynchus mykiss*) served as the donor and masu salmon (*Oncorhynchus masou*) as the recipient, resulting in the production of surrogate broodstock. Following transplantation of trout germ cells, the donor-derived cells successfully differentiated into both sperm and eggs within masu salmon recipients, demonstrating the bisexual gametogenic potential of transplanted germ cells (Okutsu et al., 2006; 2007). This capacity demonstrates that donor germ cells are able to differentiate into both male and female gametes within the host, thereby advancing the application of germ cell transplantation as a technique for the conservation of endangered or rare species. The technique has already been applied successfully across diverse teleost taxa, including marine fish such as Nibe croaker (*Nibea mitsukurii*), freshwater species like zebrafish (*Danio rerio*), and sturgeons (Takeuchi et al., 2009; Wong et al., 2011; Pšenička et al., 2015). In Siberian sturgeon (*Acipenser baerii*), germ cells derived from both ovaries and testes were transplanted into Sterlet (*A. ruthenus*) larvae, where they proliferated and established within the host. These findings highlight the potential of extending germ cell transplantation to sturgeon species, supporting its viability as a strategy for their conservation and propagation.

The sterlet (*Acipenser ruthenus*) is a relatively small freshwater sturgeon species, reaching an average body length of about 130 cm and a body weight of up to 16 kg, and is primarily distributed in the upper stretches of European rivers and their tributaries (Kalbassi et al., 2012;

Falahatkar et al., 2013). In natural habitats, males and females attain sexual maturity at approximately 5 and 7 years, respectively, whereas under aquaculture conditions, maturation occurs earlier, at around 3 years for males and 5 years for females. The International Union for Conservation of Nature (IUCN) classifies the sterlet as a vulnerable species (Pšenička & Saito, 2020). Importantly, its full life cycle from artificial propagation and larval rearing to sexual maturity has been successfully achieved under controlled aquaculture settings, enabling the annual production of tens of thousands of larvae. Furthermore, among sturgeons, the sterlet is phylogenetically the closest extant relative to the beluga (*Huso huso*) (Pšenička & Saito, 2020; Havelka et al., 2011). The great sturgeon, or beluga (*Huso huso*), is native to the Caspian, Black, and Azov Seas. Owing to its rapid growth rate and the production of caviar with exceptional nutritional and commercial value, beluga is considered a highly desirable species for aquaculture (Kalbassi et al., 2012; Falahatkar et al., 2013). With a karyotype of 118 chromosomes, it also represents an important model for genetic and evolutionary studies within the sturgeon family (Havelka et al., 2011). In natural environments, males and females typically reach sexual maturity at 12–14 and 16–18 years of age, respectively, whereas in aquaculture systems maturation occurs at a much earlier age (Kalbassi et al., 2012; Falahatkar et al., 2011). However, population declines in the wild, combined with increasing demand for meat and caviar and the considerable challenges associated with farming this species, underscore the urgent need for its conservation (Falahatkar et al., 2013). Given that the sterlet appears to be the most suitable host for supporting germ cell transplantation from beluga, the present study aimed to establish an intraperitoneal spermatogonial cell transplantation protocol using sterlet hatchlings as recipients and beluga testicular germ cells as donors. In our previous work, we developed an efficient enzymatic dissociation method for isolating beluga testicular cells and established protocols for sterilizing sterlet embryos (Borhani et al., 2022). In this study, type A spermatogonia were further purified by Percoll gradient centrifugation, identified through immunolabeling, and transplanted into newly hatched sterile sterlet larvae. Transplantation success was assessed by monitoring donor-derived cells over a two-month period and by molecular analyses performed after six months of host rearing gonads. This work outlines the essential steps toward producing beluga germline chimeras.

2. Materials and methods

2.1. Ethics Statement

All experimental procedures were approved by the Animal Research Committee of the Faculty of Natural Resources and Marine Sciences in Noor, Iran, and the Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic (facility license no. 16OZ19179/2016–17214). All methods were performed in accordance with the relevant guidelines and regulations, namely the Guidelines for the Use of Fishes in Research and Act no. 246/1992 Coll. on the Protection of Animals against Cruelty. The study is reported in accordance with the ARRIVE guidelines where applicable.

2.2. Fish and Rearing Conditions

The experiments were carried out by using Beluga (*Huso huso*) as the donor species and sterilized Sterlet (*Acipenser ruthenus*) larvae served as recipients. Beluga were reared in a

recirculating aquaculture system under standardized conditions: mean water temperature 17 °C, dissolved oxygen 7.4–7.8 mg L⁻¹, pH 7.98, and a controlled photoperiod of 16 h light and 8 h dark provided by artificial illumination. Each tank contained approximately 1000 L of water, with 20% of the volume exchanged daily. Fish were fed twice daily with a commercial extruded diet (Alcedor; 46% crude protein, 14% crude fat, 12% ash, 4% crude fiber, 10% moisture), and feeding was discontinued 72 h prior to experimental manipulation. Testicular tissue was obtained from one-year-old immature Beluga males (n = 5) following deep anesthesia with 0.1% 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222) (Sigma, St. Louis, MO, USA). The body weights of the fish ranged from 600 to 2,850 g. Based on our previous research, fish at this age had the gonadosomatic index (GSI) $0.19 \pm 0.03\%$ and their testes predominantly contained type A spermatogonia, making them suitable for germ cell collection (Borhani et al., 2022). Sterlet broodstock were propagated and sterilized as described in Borhani et al. (2021) briefly, recipient Sterlet embryos were sterile using two approaches: (i) heat shock at 37–38 °C for 2–3 min, applied 20 min after fertilization to induce triploidy, or (ii) microinjection of antisense morpholino oligonucleotides targeting the *dead end* (*dnd*) gene (Borhani et al., 2021).

2.3. Testis Cell Isolation and Purification of Spermatogonia

Testicular cell suspensions were prepared following the enzymatic dissociation protocol of Borhani et al. (2021). Briefly, approximately 0.1 g of gonadal tissue was divided into 15 tubes, each containing 50 mL of phosphate-buffered saline (PBS; Sigma–Aldrich, P4417) supplemented with 0.1% trypsin (Sigma–Aldrich, T1426). Tissues were incubated for 2–3 h at 18–20 °C. The osmolality of the medium was adjusted to match blood plasma (mean 238 mOsm kg⁻¹), and pH was set to 8. The resulting suspension was passed through a 50 µm nylon mesh (Partec, Germany) to remove debris, and 1% BSA (Sigma–Aldrich, A7511) along with DNase I (50 mg mL⁻¹; AppliChem, A3778) were added. Cell suspensions were centrifuged at 500 g for 20 min at 20 °C, and pellets were resuspended in 0.3 mL PBS without enzymes. For enrichment of spermatogonial cells, the dissociated testis cells were subjected to Percoll (Sigma–Aldrich, P1644) density gradient centrifugation. The suspension was layered onto a discontinuous gradient of 20%, 30%, and 40% Percoll in PBS and centrifuged at 800 g for 30 min. Each fraction was collected, diluted 1:10 in PBS, and centrifuged again under the same conditions. The resulting pellets were resuspended in PBS for further analyses. Isolated cells were characterized using immunofluorescence labeling. Cell yield and viability were assessed with a Bürker's hemocytometer and a Live/Dead Cell Double Staining Kit (Sigma–Aldrich, 04511). Cell numbers were counted in 20 hemocytometer squares across three replicates under an Olympus BH2 microscope at 100× magnification. For viability assessment, at least 500 cells per sample were examined using an Olympus IX83 fluorescence microscope.

2.4. Immunocytochemistry of Sorted Cells

Percoll-enriched testicular cells and somatic fin-derived cells used as negative controls, were mounted onto poly-L-lysine-coated glass slides and fixed in 4% paraformaldehyde prepared in PBS for 1 h. Following fixation, cells were rinsed with PBS and permeabilized with 0.3% Triton X-100. Samples were then washed three times in PBS (10 min each), cryoprotected by sequential incubation in 7.5% and 15% sucrose solutions for 2.5 h at 4 °C, and subsequently embedded in

7.5%, 15%, and 20% gelatin for 12 h at 37 °C. Sections were cut at –20 °C using a CM1850 cryostat (Leica, Germany) and mounted on glass slides. For immunolabeling, sections were blocked for 40 min in PBS containing 1% BSA and 0.05% Tween-20. Slides were then incubated overnight at 4 °C with an anti-DDX4 primary antibody (specific for sturgeon germ cells; Ye et al., 2016) diluted 1:300 in blocking solution. After three washes in blocking solution (10 min each), samples were incubated with a FITC-conjugated secondary antibody (anti-rabbit IgG; Sigma, F0382) diluted 1:80 in blocking buffer. Following three additional PBS washes, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 3 ng mL⁻¹, 10 min), rinsed in PBS, and mounted with Vectashield (Vector Laboratories, USA). Fluorescence signals were visualized using an Olympus IX83 inverted fluorescence microscope equipped with an ORCA R2 camera (Hamamatsu Photonics, Japan), and images were processed using CellSens software (Olympus, Japan).

2.5. Identification of Beluga Germ Cells

Reverse transcription polymerase chain reaction (RT-PCR) was performed to assess expression of the Vasa gene, a well-established germ cell-specific marker, in gonadal tissues of male and female Beluga. Somatic tissues, including caudal fin and skeletal muscle, were analyzed in parallel as negative controls to confirm germline specificity. To further evaluate the specificity of Vasa as a marker for distinguishing Beluga germ cells from those of Sterlet, gonadal and somatic tissues from five Sterlet individuals were also analyzed. Total RNA was extracted from 50–100 mg of tissue (gonads, caudal fin, and muscle) using the Ambion PureLink RNA Mini Kit (Thermo Fisher Scientific, USA), and first-strand cDNA was synthesized according to the manufacturer's instructions. PCR amplification of Vasa was performed using species-specific primers designed for Beluga based on the Vasa amino acid sequences reported in Ye et al., 2016) (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a housekeeping gene to serve as an internal control, confirming RNA integrity and successful cDNA synthesis. RT-PCR reactions were carried out in 20 µL volumes containing 20 ng of cDNA, with the following cycling conditions: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 20 s, 57 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Negative control reactions contained all PCR components and primers but lacked cDNA template. Amplified products were analyzed by electrophoresis on 2% (w/v) agarose gels and visualized under UV illumination.

Table 1. Sequence of *Vasa*, specific germ cells marker for Beluga.

Sequence (5'-3')	Region sequence	Primer names	Size (bp)
GGGAAAAATCACTCCCAGGC	F	Vasa gene specific for Beluga Germ Cells	441
AGCACTTGGAGATTTCCGCA	R		
GGGAAAAATCACTCCCAGGC	F		420
GGGGAAGTGTCTGTTCTGG	R		
AGCTCAATGGCAAGCTGACT	F		366

2.6. Transplantation of Germ Cells

Sterile Sterlet larvae, generated by either microinjection of *dnd*-MO or induction of triploidy through heat shock as described by Borhani et al. (2021), were used as transplantation recipients one week after hatching. The mean body weight of the larvae at the time of transplantation was 0.025 ± 0.005 g. In total, more than 360 larvae were analyzed: 240 received donor testicular cells, while 120 were maintained as non-transplanted controls. Freshly dissociated testicular cells from one-year-old Beluga were fluorescently labeled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma–Aldrich, USA), following the manufacturer’s instructions (final dye concentration: 2 μ L for 5 min). Labeled cells were loaded into glass microcapillaries (G-100, Narishige, Japan) prepared with a PC-10 puller and tip-polished with an EG-400 grinder to achieve a 40–50 μ m opening. Approximately 10 nL of cell suspension, containing ~10,000 cells, was injected into the peritoneal cavity close to the presumptive genital ridge of recipient larvae using a micromanipulator (M-152, Narishige) and a microinjector (CellTram Vario, Eppendorf, Germany) under fluorescence stereomicroscopy (M165FC, Leica). Recipient larvae were anesthetized at 5–6 days post-hatch with 0.001% 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222) (Sigma, St. Louis, MO, USA) and transferred into agar-coated Petri dishes for injection. Following transplantation, larvae were placed in recovery tanks and subsequently reared in 400-L tanks at 18 °C, with daily feeding on *Tubifex* worms. To assess colonization efficiency of donor cells, groups of 20 transplanted larvae were examined at 15, 30, and 60 days post-transplantation (dpt) under a fluorescence stereomicroscope (DM5000B, Leica). PKH26-positive donor cells localized within developing gonads were recorded as successful colonization events.

2.7. Molecular Genetic Test of Transplantation Success

To confirm the persistence of Beluga germ cells in Sterlet gonads, species-specific genomic DNA amplification was performed. At 180 days post-transplantation (dpt), approximately half of the transplanted recipients from each group were sacrificed, and their gonads were excised and preserved in absolute ethanol at 4 °C until further analysis. Gonads from non-transplanted Sterlet (n = 20; negative control) and from Beluga (n = 10; positive control) were collected in parallel. Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, USA). DNA quality and concentration were assessed by agarose gel electrophoresis (0.7% w/v) and spectrophotometry. Beluga-specific primers were selected based on Havelka et al. (2017) (Table 2). PCR reactions were performed in 10 μ L volumes containing 100 ng genomic DNA, 1 μ L of each primer (5 pmol), 5 μ L Taq 2 $\times$ Master Mix Red (Ampliqon, Denmark), and 3 μ L nuclease-free water. Thermocycling conditions were: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 20 s, 57 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were resolved on 2% agarose gels, visualized under UV illumination, and documented with a Quantum gel documentation system (Vilber Lourmat, Germany). A 50-bp DNA ladder (Invitrogen, USA) was used for size verification. In addition to DNA-based detection, expression of the germ cell-specific marker *Vasa* was analyzed by RT-PCR following the protocol described in Section 2.5. This analysis was performed on gonads from transplanted Sterlet (n =

30; putative chimeras), non-transplanted Sterlet (n = 15; negative control), Beluga (n = 3; positive control), and Sterlet (n = 3; negative species control).

Table 2. Primers used for the DNA amplification of Beluga.

Sequence (5'-3')	Region sequence	Primer name	Size (bp)
GATCTGAACATCAGCCACTGC	F	153-HHp	153
TACTCTGAACATCAGCCACTGC	R	153-uni	

2.8. Statistical Analysis

Prior to statistical testing, data were evaluated for normality and homogeneity of variance. Group comparisons were performed using independent-sample *t*-tests and one-way analysis of variance (ANOVA), where appropriate. A significance threshold of $p < 0.05$ was applied for all analyses. Results are expressed as mean $\pm$ standard deviation (SD). Statistical computations were carried out using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA).

3. Result

3.1. Isolation and Purification of Spermatogonial Type A Cells :

Histological examination of immature Beluga testes (stage I) with a mean gonadosomatic index (GSI) of $\sim 0.19\%$ (Borhani et al., 2022) confirmed the exclusive presence of type A spermatogonia within the seminiferous lobules. Testicular tissue was subjected to enzymatic dissociation using trypsin in PBS, and the resulting cell suspension was fractionated by Percoll density gradient centrifugation (20%, 30%, and 40%). Immunocytochemical analysis using an anti-Vasa (DDX4) antibody, a germ cell-specific marker, was employed to characterize the dissociated testicular cells. As expected, only undifferentiated type A spermatogonia displayed DDX4 immunoreactivity, consistent with their known capacity to migrate and colonize recipient genital ridges. The supernatant above the 10% Percoll layer contained mainly lipid droplets with negligible cellular material. In contrast, fractions recovered from the 20%, 30%, and 40% gradients yielded $4.2 \pm 0.76 \times 10^5$, $4.4 \pm 0.81 \times 10^5$, and $1.9 \pm 0.36 \times 10^5$ DDX4-positive cells, respectively (Table 3). No fluorescence signal was detected in dissociated somatic control cells. Based on cell yield, viability, and enrichment of DDX4-positive spermatogonia, the 20–30% Percoll fractions were identified as optimal for isolating type A spermatogonia from Beluga testes ($p > 0.05$; Table 3). On average, $\sim 0.35 \times 10^6$ DDX4-positive cells were obtained per donor male.

Table 3. Effect of different concentration of Percoll solution, for purification of Beluga type A spermatogonial, on yield and viability of testicular cells, and the number of DDX4 antibody positive cells.

DDX4 positive cell mean	Cell viability mean	Cell yield mean	Percoll solution concentration (%)
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$4.2 \pm 0.76 \times 10^{5a}$	63.79 ± 14.03^a	$7.2 \pm 0.31 \times 10^{5a}$	20
$4.4 \pm 0.81 \times 10^{5a}$	67.01 ± 7.87^a	$6.8 \pm 0.79 \times 10^{5a}$	30
$1.9 \pm 0.36 \times 10^{5b}$	58.14 ± 6.21^b	$4.3 \pm 0.54 \times 10^{5b}$	40

Data are presented as mean $\pm$ SD for three independent experiments. Values with the different superscript letters are significantly different ($p < 0.05$) as determined by One-way ANOVA test.

3.2. Vasa Expression in Different Tissues

Reverse transcription polymerase chain reaction (RT-PCR) analysis demonstrated that *Vasa* transcripts were exclusively expressed in the gonads, with amplicons of 441 bp and 420 bp corresponding to the expected product sizes. Expression was markedly higher in testes compared to ovaries, whereas no amplification was detected in somatic tissues such as caudal fin or muscle. GAPDH was used as a housekeeping gene to validate RNA quality and normalize expression levels across samples (Figure 1).

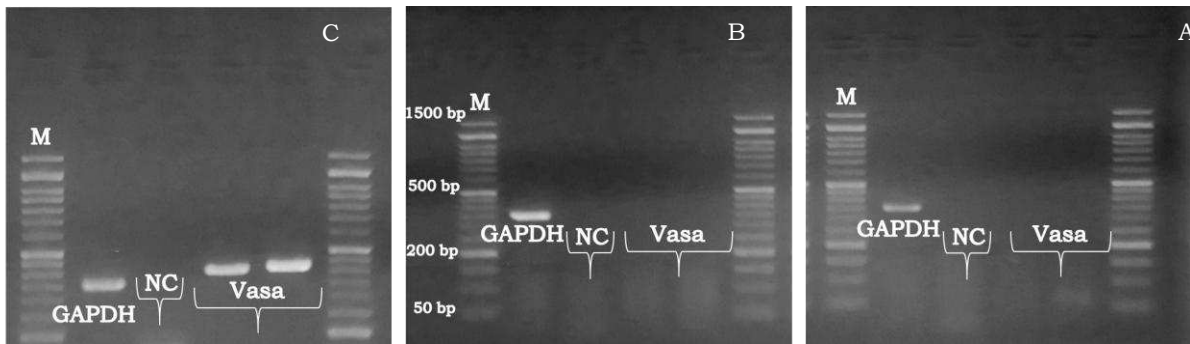


Figure 1. Electrophoresis in 2% agarose gel for RT-PCR products of the Beluga specific germ cells (*Vasa*) ($n=5$). A= muscle tissue, B= caudal fin, as control groups, and C= gonad tissue.

Vasa= reverse transcription of *Vasa* (441 and 420 bp), NC= negative control with H_2O , GAPDH=reference gene (366 bp), and M= Ladder molecular weight of 50 pb.

3.3. Transplantation of Donor Spermatogonial Cells

Following enzymatic dissociation, testicular cells were stained with the fluorescent membrane dye PKH26 to enable transient tracing in the recipient body cavity (Figure 2). The average yield of labeled cells was $19.2 \pm 0.8 \times 10^5$, with a mean viability of $81.31 \pm 2.21\%$, which did not differ significantly from non-labeled cells ($p > 0.05$). Transplantation was performed by introducing the needle tip into the peritoneal cavity of sterile Sterlet larvae (5–6 days post-hatch, dph) in both *dnd-MO* treated and triploid ($3n$) groups. The injection site was selected near the posterior intestine, corresponding to the endogenous location of primordial germ cells (PGCs) in Sterlet (Saito et al., 2014). Fluorescence microscopy confirmed successful delivery of PKH26-labeled donor cells into the peritoneal cavity of both recipient groups (Figure 3a-1, b-1, c-1), while no fluorescent signal was observed in non-transplanted controls (Figure 3a-2, b-2, c-2).

Cell tracing demonstrated progressive colonization of donor cells within recipient gonads over time. At 15 dpt, donor cells were detected in >70% of recipients (Table 4), primarily dispersed in the abdominal cavity (Figure 3a-1). By 30 dpt, cells were observed in 90% of recipients, with some showing weak or tight attachment to the abdominal wall (Figure 3b-1). At 60 dpt, donor cells were detected in >80% of recipients, where they became organized into genital ridges (Figure 3c-1). Quantitative assessment revealed that the number of labeled donor cells at 60 dpt was significantly higher than at 15 or 30 dpt ($p < 0.05$), indicating active proliferation following transplantation. Moreover, colonization efficiency was significantly greater in *dnd-MO* treated recipients compared with the 3n group ($p < 0.05$).

Molecular confirmation of donor cell colonization was obtained by PCR amplification of *Beluga*-specific genomic DNA from recipient gonads at 180 dpt. Specific amplicons were detected in 62.98% of *dnd-MO* treated recipients and 40.45% of 3n recipients, whereas no amplification was observed in non-transplanted controls (Figure 4). RT-PCR analysis of the *Vasa* germ cell marker further supported these findings; expression was detected in 52% of transplanted gonads overall, with a significantly higher frequency in *dnd-MO* treated recipients (58.26%) compared to 3n recipients (46%) ($p < 0.05$; Figure 5). No *Vasa* expression was observed in non-transplanted individuals. Finally, survival analysis revealed group-dependent differences. The survival rate of *dnd-MO* treated recipients ($77.67 \pm 6.19\%$) was significantly lower than that of 3n recipients ($92.5 \pm 4.40\%$) and non-transplanted controls ($91.81 \pm 3.09\%$) ($p < 0.05$).

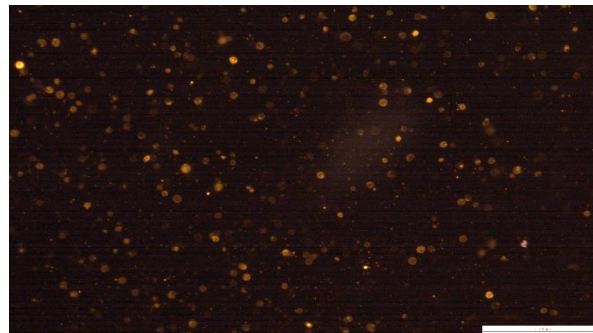
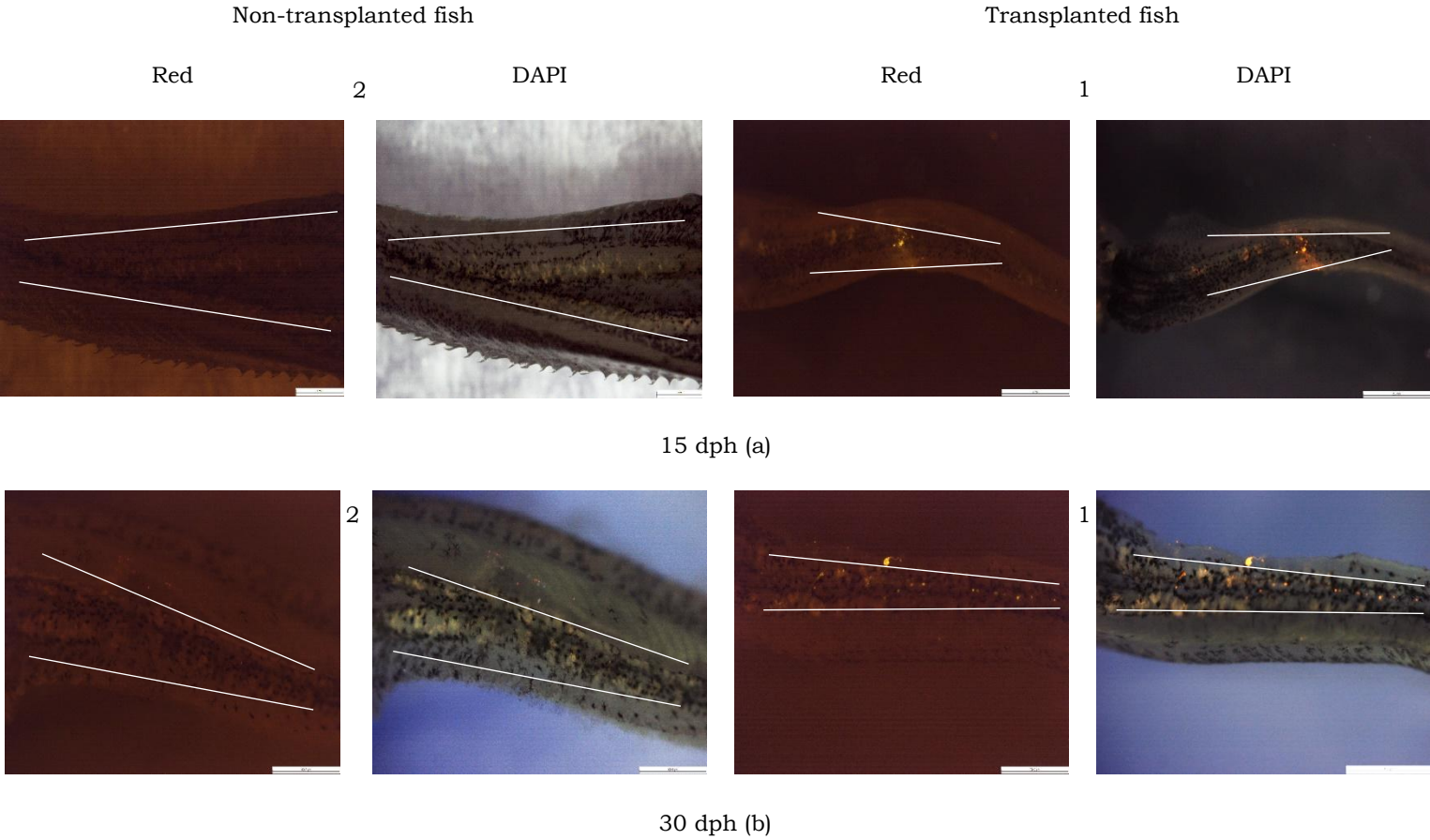


Figure 2. Plasma membrane, Beluga spermatogonial cells, was labelled with PKH26 fluorescent dye, scale bar is 550 μm .

Table 4. The success of Beluga spermatogonial cells transplantation into sterile Sterlet groups.

Non-transplanted fish (control)		Transplanted fish 3n recipient		Transplanted fish <i>Dnd-MO</i> treated recipient		Time days post transplantation (dpt)
Number of cells, mean $\pm$ SD	Positive fish	Number of cells mean $\pm$ SD	Positive fish	Number of cells mean $\pm$ SD	Positive fish	
-	0	14.27 $\pm$ 4.82 ^a	14/20	12.57 $\pm$ 3.42 ^b	15/20	15 dph
-	0	14.45 $\pm$ 4.62 ^a	16/20	14.85 $\pm$ 4.51 ^b	20/20	30 dph
-	0	25.34 $\pm$ 15.63 ^b	16/20	36.05 $\pm$ 19.81 ^a	17/20	60 dph

Table shows the success of transplantation and average number of transplanted Beluga spermatogonial cells in time with standard deviation in positive samples. Values with the different superscript letters are significantly different (ANOVA, $p < 0.05$). Abbreviations: dpt, days post transplantation; SD, standard deviation.



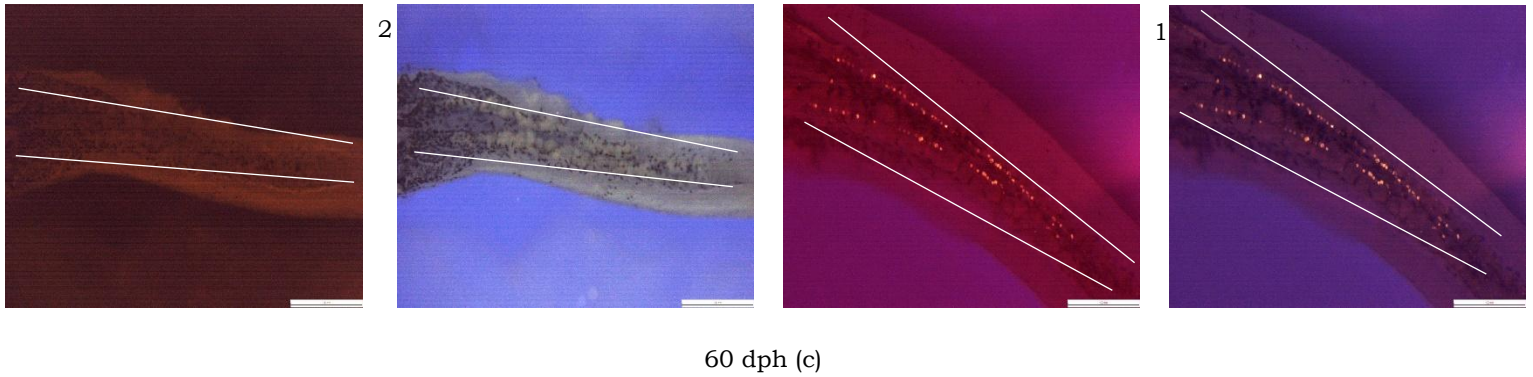


Figure 3. Xenotransplantation of Beluga spermatogonial cells into newly hatched sterile Sterlet. Detection of the fluorescently labelled germ cells within the recipient gonads (delineated by white lines) signified successful incorporation of the donor-derived germ cells. a-1, b-1, c-1= Sterlet genital ridge with transplanted Beluga spermatogonial cells. (a-1) Sterlet with PKH26-labeled (red) at 15 days post transplantation (dpt) (scale bar 2.5 mm), (b-1) 30 (scale bar 1 mm), and (c-1) 60 dpt (scale bar 1.2 mm). a-2, b-2, c-2= genital ridge of non-transplanted Sterlet as control group at 15, 30, and 60 dph (scale bar 2.5, 1, and 1.2 mm, respectively).

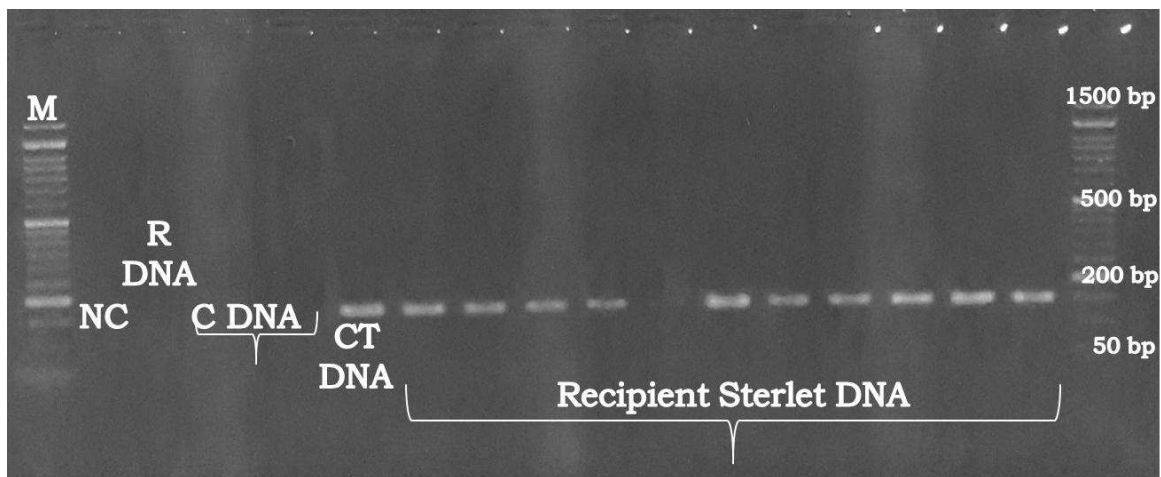


Figure 4. Electrophoresis in 2% agarose gel for amplicons of the genomic DNA (HHp153+uni153) at 180 dph by PCR. Recipient Sterlet DNA= of transplanted Sterlet with Beluga spermatogonial cells, CT DNA= positive control of Beluga DNA, C DNA= control groups DNA or non-transplanted Sterlet, R DNA= Negative control of Sterlet DNA, NC= negative control with H₂O, M= . Ladder molecular weight of 50 pb.

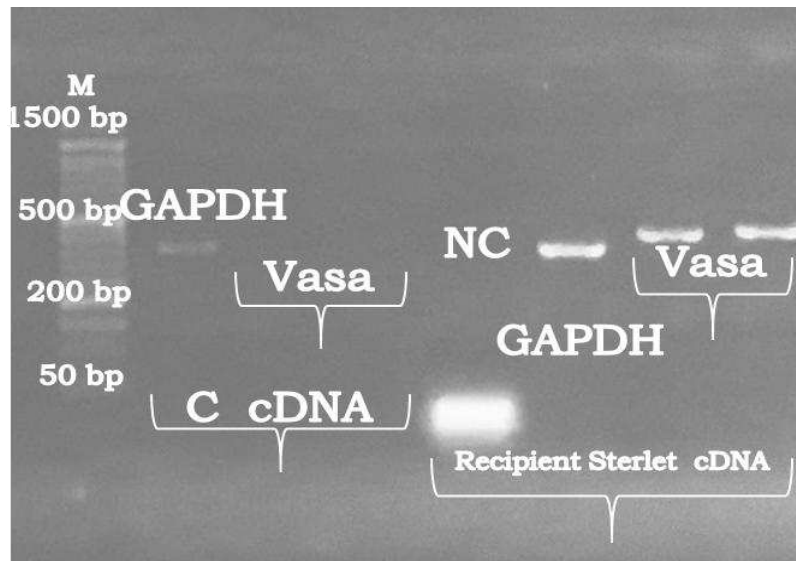


Figure 5. Electrophoresis in 2% agarose gel for RT-PCR products of the Beluga specific germ cell marker, *Vasa*, in transplanted gonads fish at 180 dph. *Vasa*= cDNA from transplanted Sterlet for *Vasa* (441 and 420 bp), C cDNA= cDNA from control group or non-transplanted Sterlet, GAPDH= Reference gene (366 bp) for both transplanted and non-transplanted Sterlet, NC= negative control with H₂O, and M= Ladder molecular weight of 50 pb.

4. Discussion

In sexually reproducing organisms, primordial germ cells (PGCs) represent the only embryonic cell population capable of transmitting genetic information to the next generation. These cells undergo proliferation, migrate to the genital ridges, and eventually differentiate into functional gametes (Brinster, 2002; Okutsu et al., 2006; Kawakami et al., 2010; Yamaha et al., 2010). Owing to their inherent migratory ability, PGCs—and their later developmental derivatives, such as spermatogonia and oogonia are particularly suitable for germline chimera induction via transplantation (Saito et al., 2008). Compared with PGCs, spermatogonia (SG) and oogonia (OG) present practical advantages. They can be isolated directly from donor gonads in large quantities, and these cells can be transplanted into the peritoneal cavity of recipient fry without losing their colonization ability, even in cases where migratory efficiency is suboptimal (Yoshizaki et al., 2010; Pšenička et al., 2015). Thus, germ cell transplantation represents a powerful biotechnological tool for the creation of germline chimeras. Importantly, this approach has significant conservation potential, enabling the propagation of endangered species through closely related but more manageable surrogate hosts (Okutsu et al., 2006; Yamaha et al., 2007). The application of germline chimera technology offers several key benefits: (1) shortening reproductive cycles of species with long maturation periods; (2) reducing the rearing space required when smaller host species are used; (3) enabling long-term preservation of genetic resources through germ cells rather than live broodstock; and (4) minimizing the need to maintain large, slow-maturing adults of endangered species (Yamaha et al., 2010; Linhartová et al., 2014).

In this study, we selected Sterlet (*Acipenser ruthenus*), a small-bodied sturgeon species with a relatively short maturation time (~5 years), as a recipient host for germ cell transplantation. The donor species, Beluga (*Huso huso*), is critically endangered and characterized by a very long

reproductive cycle, requiring approximately 18 years to reach sexual maturity. By establishing germline chimerism between these two species, it may be possible to accelerate Beluga gamete production nearly four-fold compared to natural maturation. This strategy highlights the potential of surrogate propagation as a conservation approach for sturgeons, combining practicality, genetic resource preservation, and recovery of species threatened by overfishing and habitat degradation.

Enrichment of early-stage male germ cells in the present study was effectively achieved using 20–30% Percoll gradient centrifugation. However, a considerable proportion of somatic cells remained in the suspension, indicating that further refinement of the purification strategy is required. An alternative and more selective method has been proposed by Von Schönfeldt et al. (1999), who demonstrated the enrichment of spermatogonia in several mammalian models through magnetic-activated cell sorting (MACS) based on specific surface markers of spermatogonial stem cells. Such marker-based separation techniques may offer a higher degree of purity compared with density-gradient centrifugation and could be considered for future sturgeon germ cell studies.

Panda et al. (2011) successfully applied this technique in the cyprinid fish *Labeo rohita*. However, the application of similar approaches in sturgeons remains limited due to the absence of germ stem cell-specific surface markers (Pšenička et al., 2015). In the present study, germ cells were identified by their expression of the evolutionarily conserved germline marker Vasa. Previous investigations, such as those of Rzepkowska and Ostaszewska (2014), have demonstrated Vasa protein localization in germline cells during early gonadal development of cultured Siberian and Russian sturgeon. Consistently, our RT-PCR analysis revealed Vasa-specific transcripts exclusively in gonadal tissues, while purified cells were further validated by positive DDX4/Vasa immunolabeling, confirming their germline identity in beluga. The use of Vasa as a reliable germ cell marker has also been documented in multiple teleost species (Lacerda et al., 2014; Pšenička et al., 2015; Poursaeid et al., 2020).

Following Percoll gradient separation, the proportion of Vasa-positive spermatogonia obtained at 20% and 30% fractions was comparably high ($p > 0.05$), confirming the efficiency of these concentrations for enrichment. On average, 0.35 million DDX4-positive cells were recovered from a single beluga individual. This relatively high yield is attributable to the large gonadal size and delayed sexual maturation characteristic of sturgeons. In contrast, Wong et al. (2011) reported only ~755 Vasa-positive ovarian cells isolated from individual zebrafish, highlighting the substantial advantage of using sturgeon as a donor species in germ cell transplantation studies.

Since only germline stem cells (SSCs) have the intrinsic ability to colonize recipient genital ridges and subsequently reinitiate donor-derived gametogenesis, spermatogonial transplantation represents a functional assay to evaluate the presence of SSCs within a donor cell population (Lacerda et al., 2014). In this study, freshly isolated beluga spermatogonial cells obtained by enzymatic dissociation were intraperitoneally xenotransplanted into newly hatched sterile sterlet larvae belonging to a different genus. Fluorescent tracing of PKH26-labeled donor cells over a two-month period demonstrated that a subset of transplanted cells retained SSC properties, including migration from the peritoneal cavity, the injection site, to the genital ridges of recipient sterlet, where they established colonization. These observations confirmed that the cell suspension contained functional SSCs. Confocal microscopy of recipient gonads at

15, 30, and 60 days post-transplantation (dpt) revealed colonized PKH26-labeled cells with morphological features consistent with donor spermatogonia. A progressive increase in transplanted cell number was observed by 60 dpt (equivalent to 67 days post-hatching), indicating active proliferation. This timing aligns with the onset of proliferation and differentiation reported in Siberian and Russian sturgeon, which typically occurs around 115 days post-hatching (Rzepkowska & Ostaszewska, 2014).

Although transplanted cells in both sterile recipient groups exhibited migration, localization, and proliferation, differences emerged between treatments. In some triploid (3n) sterlet, donor cells remained confined to the injection site even at 60 dpt, whereas in *dnd-MO* treated sterlet, transplanted cells successfully migrated to the genital ridges and established colonization. These results indicate that the sterlet somatic microenvironment supports beluga SSC survival, but *dnd-MO* induced sterilization provides a more permissive niche for donor-derived gametogenesis compared to triploidy. This observation is consistent with Linhartová et al. (2015), who showed that eliminating primordial germ cells (PGCs) via *dead end* knockdown enhances donor germ cell colonization. Molecular analyses further confirmed these findings. PCR and RT-PCR assays using beluga-specific primers verified the persistence of donor cells within the gonads of both sterile recipient groups. However, colonization and maintenance rates were significantly higher in *dnd-MO* recipients, likely because triploid sturgeon retain partial fertility (Havelka et al., 2014), which may hinder complete donor cell integration. Despite its effectiveness, the *dnd-MO* sterilization method presents practical challenges. Microinjection of morpholino antisense oligonucleotides must be performed at early cleavage stages under precise micromanipulation, requiring specialized expertise and equipment. The procedure is labor intensive, time sensitive, and costly, with potential egg toxicity at higher morpholino concentrations (Linhartová et al., 2015; Borhani et al., 2021). Nevertheless, our two-month monitoring of cell proliferation combined with molecular validation at both DNA and RNA levels supports the conclusion that *dnd-MO* treated sterlet represent suitable and efficient recipients for beluga SSC transplantation.

The first successful xenotransplantation of spermatogonial cells in sturgeons was reported by Pšenička et al. (2015), where sterlet (*Acipenser ruthenus*) recipients were able to produce both eggs and sperm of Siberian sturgeon (*A. baerii*). More recently, Ye et al. (2017) demonstrated that germ cells from Chinese sturgeon (*A. sinensis*) could successfully incorporate into the gonads of Dabry's sturgeon (*A. dabryanus*) larvae. Collectively, these studies highlight the absence of strict phylogenetic barriers among sturgeon species, supporting the idea that germ cell migration and colonization are evolutionarily conserved processes within this lineage. In the present study, we showed that beluga spermatogonial cells can be efficiently enriched using a Percoll density gradient of 20–30%. Following purification, xenotransplantation of these cells into the peritoneal cavity of sterile sterlet recipients treated with *dnd-MO* resulted in efficient migration, colonization, and proliferation within the recipient gonads. These results demonstrate that sterlet larvae can serve as practical surrogates for beluga, enabling donor derived germ cell production within a shorter time frame compared to the natural maturation period of beluga.

The approaches described here provide a valuable platform for germplasm preservation and gene banking of endangered sturgeons. Moreover, this system may be extended to aquaculture

biotechnology, offering potential applications in the production of gametes from late maturing sturgeon species, thereby supporting both conservation and sustainable fish farming.

5. Limitations

A major limitation of this study was the limited availability of Beluga donor fish. Because Beluga is a valuable and endangered species, the number of fish available for destructive sampling was restricted, and donor selection had to be carefully timed according to age and gonadal developmental stage to obtain enough germ cells. Despite this limitation, the present findings demonstrate successful migration and colonization of Beluga germ cells in Sterlet recipients.

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Declaration of Competing Interest

The authors declare no competing financial or personal interests that could have influenced the work reported in this paper.

Data Availability Statement

The datasets generated and analysed during the current study are available in the Zenodo repository, <https://doi.org/10.5281/zenodo.21970521>

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