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Micromanipulation and cryopreservation of germ cells in fish

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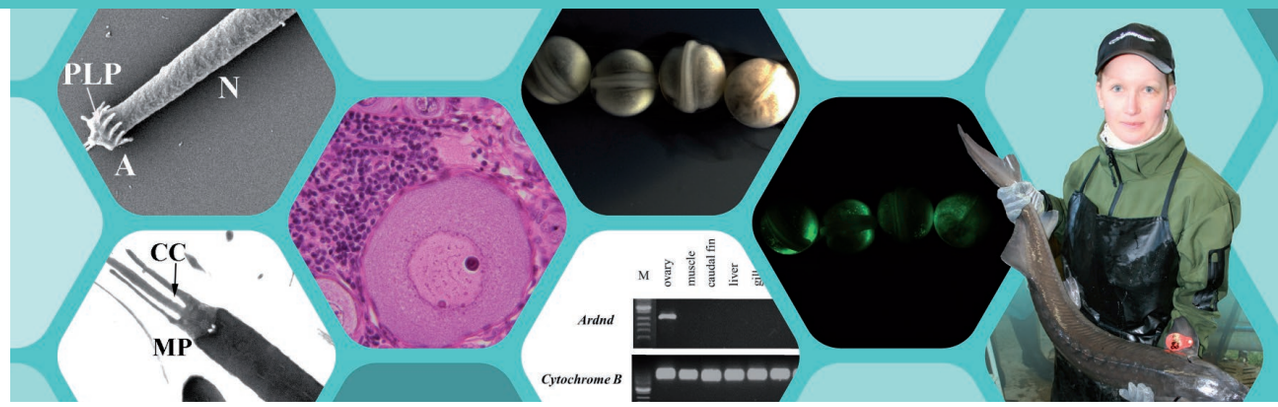

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1. Importance of early stages of germ cells

Primordial germ cells (PGCs, precursors of germ cells) are the only cells in developing embryos with potential to transmit genetic information to the next generation (Kawakami et al., 2010). Then these cells differentiate into spermatogonia (SG)/oogonia (OG), which are the first germ cells of testes/ovary. PGC/SG/OG, altogether called as early stages of germ cells (GC), have great potential for use in gene banking and cryopreservation, particularly through the production of germ-line chimeras producing donor´s gametes (Chapter 1.6.) (Okutsu et al., 2006a, b; Yamaha et al., 2007).

Before PGCs are used for this purpose, first, their origin, character and migration pathway needed to be defined. In most organisms, the PGCs migrate over long distances and follow a complex of path (Saito et al., 2014). Their migration activities are also associated with the distribution of yolk in the egg and in relation to the two different types of egg cleavage in fishes, meroblastic (partial, teleosts) and holoblastic (total, chondrostean and anurans) (Fig. 1.). PGCs´ migration has been studied extensively in *Drosophila melanogaster, Xenopus laevis*, chick *Gallus gallus domesticus* and mouse *Mus musculus* (Starz-Gaiano and Lehmann, 2001) and several teleost species (Saito et al. 2008, 2010, 2011). Saito et al. (2008) documented the PGCs´ migration from pearl danio *Danio albolineatus*, goldfish *Carassius auratus*, loach *Misgurnus anguillicaudatus* and Japanese eel *Anguilla japonica* by injecting an artificially synthesized mRNA with green fluorescent protein (GFP-*nos* 1 3'UTR mRNA) into cleaving embryos and demonstrated the differences in migratory activity of PGCs within teleosts. Moreover, they isolated these cells, transplanted a single PGC into zebrafish *Danio rerio* and documented their migration route in the host fish. Little is known about PGCs´ origin and migratory activity in chondrostean fish. Saito et al. (2014) demonstrated that the sturgeon egg has holoblastic cleavage and that the PGCs generated by the inheritance of germ plasm is maternally deposited at the vegetal pole, similar to that in anurans. Then PGCs migrate on the yolky cell mass toward the gonadal ridge, resembles the migration pattern of teleost. Taken together, the mechanism of PGCs´ migration is very conservative in different fish species and has to be properly studied before transplantation. After these studies germ-line chimera can be produced by donor GC transplantation to the host.

Development of primordial germ cell transplantation techniques (Saito et al. 2008, 2010) was based on their ability of migration to genital ridge. Another possibility of PGCs transplantation is the transplantation of blastomeres containing PGCs. This has been performed in different fish species including zebrafish (Lin et al., 1992), medaka *Oryzias latipes* (Wakamatsu et al., 1993), rainbow trout *Oncorhynchus mykiss* (Takeuchi et al., 2001), goldfish *Carrasius auratus* (Yamaha et al., 2001), and loach (Nakagawa et al., 2002). The migratory characteristic was assumed to be lacking in early stages of germ cells, the SG/OG (Raz and Reichman-Fried 2006), but this has been refuted by Okutsu et al. (2006a, b) and Yoshizaki et al. (2010), who argue that SG exhibit at least a low level of migration activity in response to chemotactic signals. The advantage of SG/OG compared with PGCs is in the greater ease in isolating them from testes/ovary resulting in a higher yield of cells which can be directly microinjected into the body cavity of recipient fry and to counteract the potential inability of transplanted GC to migrate (Yoshizaki et al., 2010; Psenicka et al., 2015). Therefore, an alternative method of PGCs transplantation is the transplantation of SG/OG by microinjection into the body cavity of recipient.

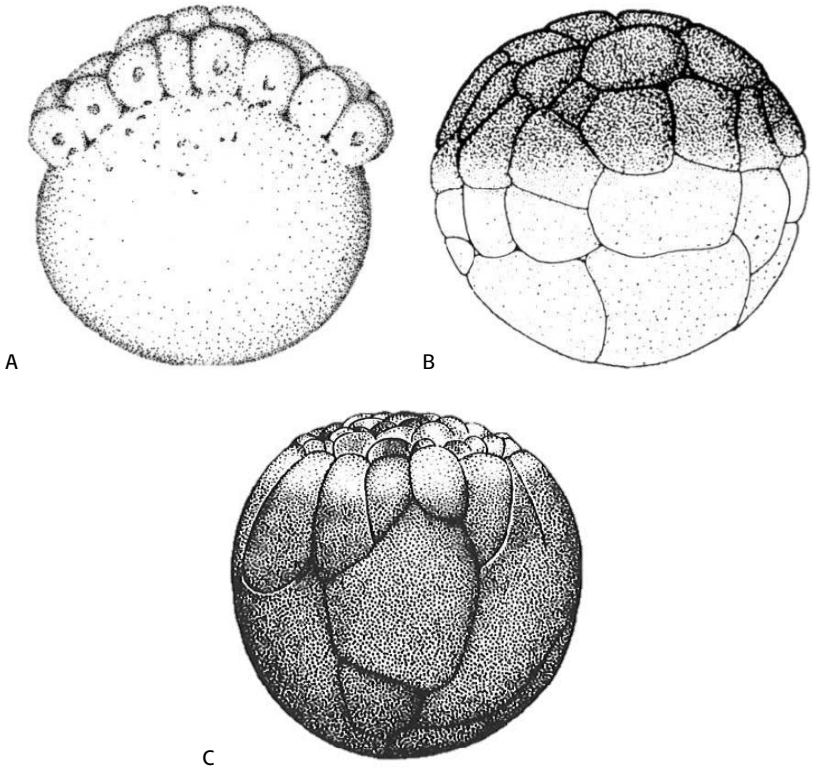

Fig. 1. *Different egg cleavage types of three vertebrate species: A – zebrafish cleavage – a meroblastic cleavage typical for many teleots, B – Xenopus cleavage – a holoblastic cleavage typical for anurans, C – sturgeon egg cleavage – a holoblastic cleavage as seen in anurans (Detlaff et al., 1993).*

1.2. Fish of interest

All of my studies involved fishes from two different subclasses. The first species is from the infraclass *Teleostei*, order *Cypriniformes*, the tench *Tinca tinca*, and the second is from subclass *Chondrosteii*, order *Acipenseriformes* (sturgeons and paddlefishes) with two species represented by sterlet *Acipenser ruthenus* and beluga *Huso huso*.

The tench is a cyprinid species related to carp *Cyprinus carpio*, goldfish, zebrafish, and white cloud mountain minnow *Tanichthys albonubes* (Nelson, 2006). In recent years, the demand for tench fry and stock for aquaculture has been increasing, and research has been conducted on all aspects of their artificially controlled reproduction (Podhorec et al., 2011). The cryopreservation of tench sperm (Rodina et al., 2007) or germ cells (present study) may directly benefit artificial reproduction. Therefore, tench, an economically important fish, has been traditionally reared in ponds of Central and East Europe (Gela et al., 2007), Asia (Wang et al., 2006) and is also being cultured in North America and Africa (Nelson, 2006), was chosen as a species of interest for this project. Moreover, due to mentioned relationship with small-bodied zebrafish or minnow, this species can possibly be used as a host for xenogeneic transplantation (Chapter 1.6.).

Perhaps there is a greater general interest in the second subclass, the sturgeons. With the recent worldwide increase of caviar consumption and poaching of natural populations, construction of dams, continuing damage to the natural habitats, and environmental pollution,

these fishes are listed in IUCN Red List of Threatened Species as the most endangered group of species in the world (http://www.iucnredlist.org/news/sturgeons-highly-threatened). Their artificial reproduction is complicated by their reproduction characteristics such as late maturation and the inability of females to repeat the reproduction every year (Havelka et al., 2011). However, even if genetic and cytogenetic manipulations in sturgeons are very difficult compared to teleost species, it is of interest to make the manipulations and cryopreservation of germ cells for sturgeon reproduction and conservation more efficient (Saito et al., 2014). We focused on sterlet *Acipenser ruthenus*, which is the most common and smallest Eurasian sturgeon species with fastest maturation and shortest reproductive cycle (sexual maturity of males 3–7 and females 5–9 years of age) in comparison with other sturgeons (Detlaff et al., 1993). We were also interested in beluga *Huso huso* which is among the largest fresh water fish with longest maturation or reproduction cycle (maturation 15–20 years) among sturgeons. Furthermore, the study of spermatozoa structure provided important information to increase knowledge of evolutionary and taxonomic relationships among family and genera of *Acipenseriformes* (Jamieson, 1991; Mattei, 1991; Lahnsteiner and Patzner, 2008; Linhartova et al., 2013).

1.3. Micromanipulation with germ cells

The micromanipulation biotechnology was firstly used for gene transfer to generate genetically modified organisms (GMO). The gene construct (gene + promoter) with suitable vector uses micromanipulations to import the construct to the target organism. Under optimal conditions, modified transgenic genetic information of an individual is transmitted to next generation. These genetic modifications are commonly used in studies of gene expression as well having practical use in agriculture. In fisheries, these technologies are used to improve characteristics: intensity of growth, disease resistance, cold resistance, changes in metabolism, sterilization etc. (Flajshans et al., 2013). The preliminary experiments (for future transplantation of germ cells to induce germ-line chimera) were aimed to optimization of visualization of PGCs or SG/OG, identification of PGCs or SG/OG and effective isolation of these cells.

The visualization of PGCs is the first important step of germ-line chimera construction. The most common method for germ cell tracing uses a green fluorescent protein (GFP) which is linked to a complementary sequence of the 3'UTR of zebrafish *nanos* (*nos* 1) or the 3'UTR of medaka *Oryzias latipes vasa* homologue (*olvas*) (Köprunner et al., 2001; Kurokawa et al., 2006). PGCs have been visualized in several fish species of salmonids and cyprinids by microinjection of similar mRNA construct into fertilized eggs of the rainbow trout or zebrafish (Yoshizaki et al., 2005). Saito et al. (2006) tested the efficiency of PGCs labeling by GFP-*nos* 1 3'UTR mRNA and GFP-*olvas* 3'UTR mRNA in a variety of fish. To investigate the relationship between phylogenetic distance and function of the 3'UTR of zebrafish *nanos* or medaka *vasa* genes, they compared seven teleost species: herring *Clupea pallasii*, zebrafish, pearl danio, goldfish, loach, medaka and ice goby *Leucopsarion petersii.* They demonstrated, that PGCs of pearl danio, goldfish, loach, herring, medaka and ice goby could be visualized using GFP-*nos* 1 3'UTR and suggested that the function of the *nos* 1 3'UTR is highly conserved in teleosts. GFP-*olvas* 3'UTR mRNA did not identify PGCs in this species besides medaka (Saito et al., 2006).

We have followed this method for tench PGCs tracing (Saito et al., 2006, 2008) and injected the GFP-*nos* 1 3'UTR mRNA into the blastodisc of fertilized eggs at 1- to 4-cell stage. After injection of this probe, we observed the development and migration route of PGCs in tench embryos during their embryonic and larval development and documented basic information

about PGCs migration pattern and early gonadal development (Linhartova et al., 2014a – Chapter 3). Moreover, Saito et al. (2014) used the same technique to trace the origin and migration routes of sturgeons PGCs´.

1.4. Isolation and cryopreservation of germ cells

The importance of preserving the genetic resources has increased substantially. Cryopreservation and cryobanking of fish gametes are considered tools for artificial reproduction and genetic improvement of aquaculture species (Alavi et al., 2012). This process in fish spermatozoa is relatively simple (Bobe and Labbe, 2010) and has been applied in more than 200 fish species (Billard and Zhang, 2001). The disadvantage of sperm cryoconservation is that it does not provide access to maternal information. For fish, the successful cryopreservation of the gametes is currently possible only for spermatozoa. Fish oocytes and embryos are difficult to cryopreserve because of their large size, high yolk content which results in slow penetration of the extender, and high sensitivity to freezing (Zhang et al., 2007; Robles et al., 2009). The most effective alternative for the female genome is the use of early stages of germ cells, is using OG from the ovary and SG from testes; these germ cells have the capability to re-colonize genital ridges upon transplantation (Yoshizaki et al. 2005, 2011; Okutsu et al. 2006b). GC are well-suited to cryopreservation, due to small size and a high level of sexual plasticity which allows them to differentiate into fully functional gonads of both sexes and produce functional eggs and/or sperm (Okutsu et al., 2006b, 2007). The aim of this part of study was to investigate the optimal procedure for tench and sturgeon GC enzyme dissociation, isolation and cryopreservation, focusing on cryoprotectant solution.

Isolation of germ cells

The efficiency of isolation is facilitated by enzyme-induced dissolution of the germ cells from testicular/ovarian tissue and by the isolation technique (Bellve et al. 1977). These authors dissociated the SG using 0.1% collagenase and 0.25% trypsin in two steps by separation of GC according to sedimentation speed in a concentration gradient of 2-4% bovine serum albumin (BSA). Lacerda et al. (2006) dissociated testes of sexually mature Nile tilapia *Oreochromis niloticus* with collagenase and trypsin in the same way as Bellve et al. (1977), sorted SG from testicular cells by a Percoll gradient and transplanted the cells into urogenital papilla. Okutsu et al. (2006b) isolated SG from adult male rainbow trout for the first time and performed a xenogeneic SG transplantation from rainbow trout to the masu salmon *Oncorhynchus masou*. The trout testes were incubated and dissociated in phosphate buffer saline (PBS) with 0.5% trypsin and transplanted into the peritoneal cavity of masu salmon. They showed that donor testicular germ cells can colonize host gonads at an embryonic stage and that adults can produce donor functional sperm and eggs (Okutsu et al., 2006b). Moreover, isolation of trout OG has been performed by L-15 containing 0.2 % collagenase (Yoshizaki et al., 2010). The OG isolation was also described in zebrafish by Wong et al. (2011) by dissociating ovarian tissue in PBS containing 0.2% collagenase. Linhartova et al. (2014b) reported for the first time, the dissociation and isolation of male tench GC, including SG and spermatocytes. Testicular cells were dissociated with 0.1% each of collagenase and trypsin (248 $mOsm.kg^{-1}$, pH 8) and separated by Percoll gradient. Psenicka et al. (2015) conducted isolation and transplantation of female and male GC in sturgeon. Isolation of early GC, characterized by mostly SG from testes and OG and pre-vitellogenic oocytes from ovary, of 2–4-year old Siberian sturgeon *Acipenser baerii* was performed by using 0.3% trypsin (2 h, 23 °C) dissolved in PBS, isotonic with blood plasma. The dissociated GC were sorted by Percoll gradient centrifugation. Sorted germ cells were transplanted into a body cavity close to a presumptive genital ridge of newly

hatched sterlet larvae. The transplanted donor GC successfully colonized sterlet genital ridges at 50 days post transplantation (dpt) and proliferated 90 dpt.

Cryopreservation of germ cells

Significant inter-species variations, as well as significant within-species differences in cryoprotectant effectiveness, have been reported in fish spermatozoa (Alavi et al., 2012). Therefore for optimal cryopreservation, the cryoprotectant solution and freezing method has to be properly evaluated. Fish GC cryopreservation has been successfully established in rainbow trout (Okutsu et al., 2007), Siberian sturgeon (Psenicka et al., 2012) and tench (Linhartova et al., 2014b) under similar freezing conditions. Cryomedium containing 1.8M ethyleneglycol (EG), 0.5% bovine serum albumin (BSA), and 5.5 mM d-glucose (Yoshizaki et al. 2011) resulted in 45.4% survival rate of GC of rainbow trout after thawing (Okutsu et al. 2007). Higher survival was obtained by using 1.5 M glycerol (GLY) for tench GC (Linhartova et al., 2014b). In a study of cryopreservation of GC of Siberian sturgeon, 1.5M EG was found to be the most effective cryoprotectant (Psenicka et al., 2012). Another possibility of cryopreservation is to freeze pieces of testes/ovary and dissociate germ cells after thawing (in process of thawing) according to Lee et al. (2013). Taken together, isolation and cryopreservation of GC appears to be a useful and simple technique for conservation of genetic resources in fish. The effectiveness of the extender and cryoprotectant is species-specific as well as individually dependent, and it has to be optimized for different fish species. These data are also considered preliminary results for application in fish bioengineering by transplanting cryopreserved GC into a sterile xenogeneic recipient.

1.5. Sterilization of fish

To generate germ-line chimera which will produce only the donor´s gametes, the prerequisite is the sterilization of host (sterlet and tench in present study). Triploidization or hybridization are usual methods for sterilization, as triploid or hybrid species are reported to be sterile, whereas excluding (knockdown) of any of the known genes responsible for PGCs development and migration (as *dead end*, *vasa* or *nanos* genes) is another approach. Triploidization is the method of choice for practical use in species whose 3-N individuals showed to be sterile (Vandeputte et al., 2009) but, this approach is not usable for sturgeons. Sturgeons differ in ploidy levels which is probably the result of several genome duplications (Peng et al., 2007), and at least three independent polyploidization events during evolution (Havelka, 2013). Also allopolyploidization as well as autopolyploidization seems to be an ongoing process in sturgeon with observed abnormal ploidy levels in these populations. Moreover, the specimens with abnormal ploidy level as a result of autopolyploidization showed to be fertile (Drauch et al., 2011; Havelka et al., 2014). That is why this method is not suitable for sturgeon sterilization. Similar examples are provided by interspecific hybridization of fishes. Generally in fish, interspecific hybridization of distantly related parental species results in sterility by incorrect pairing of their chromosomes (Piferrer et al., 2009). This can be useful tool for tench sterilization but not for sturgeons, because hybrids between sturgeon species with same ploidy levels are considered to be fertile (Havelka, 2013). Moreover the possible fertility of species with different ploidy level was demonstrated (Flajshans and Vajcova, 2000; Havelka, 2013). Another method of fish sterilization is by chemical treatment in form of baths or diets (e.g. busulfan) that inhibit gametogenesis at different developmental stages, but this doesn´t guarantee 100% efficiency (Lacerda et al., 2006). Taken together, polyploidization, hybridization and also chemical treatment are not useful methods of sturgeon sterilization. Non-transgenic knockdown (KD) strategy to inhibit the functions of genes involved in early

gonadal development has been suggested as suitable for sturgeon sterilization. The best candidate gene selected for KD, expressed in germ plasm, is *dead end* (*dnd*) gene (Weidinger et al., 2003). *Dnd* encodes an RNA-binding protein crucial for migration and survival of PGCs. It is a maternally inherited gene expressed in male and female gonads (Weidinger et al., 2003). Therefore, unfertilized eggs were selected as suitable material for RNA extraction (Linhartova et al., 2015, Chapter 6). The *dnd*-orthologs has already been described in invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Weidinger et al., 2003), and vertebrates such as chicken (Aramaki et al., 2007), *Xenopus laevis* (Weidinger et al., 2003), mouse (Youngren et al., 2005). In fish, it was first identified in zebrafish (Weidinger et al., 2003) and subsequently in other teleosts such as medaka (Liu et al., 2009), loach (Fujimoto et al., 2010), goldfish (Goto et al., 2012), Atlantic salmon *Salmo salar* (Nagasawa et al., 2013), Pacific bluefin tuna *Thunnus orientalis* (Yazawa et al., 2013), turbot *Scophthalmus maximus* (Lin et al., 2013) and the chondrostean species of Chinese sturgeon *Acipenser sinensis* (Yang et al., 2014) and Russian sturgeon *Acipenser gueldenstaedtii* (unpublished data). All these studies have suggested that *dnd* plays one of the main roles in germ cell development and is a useful germ cell marker for tracing their development. Moreover, its KD led to the loss of PGC migration functions and their death (Weidinger et al., 2003). Currently, one of the best KD applications that has been used in studies of developing embryos (Summerton, 2007) is morpholino antisense oligonucleotide (MO). MO is synthetic oligonucleotide used to temporary eliminate the function of genes by blocking pre-mRNA splicing or mRNA translation (Fig. 2.), so it generates non-transgenic genetically modified organisms (Draper et al., 2001; Summerton, 2007). MO is designed from ATG region of the gene to be KD. Therefore, to be able to construct MO, the sequence of target gene in start codon region has to be known. Based of known sequence of target gene, MO can be designed, synthetized and injected in germ plasm region of developing embryos for their sterilization (Chapter 6).

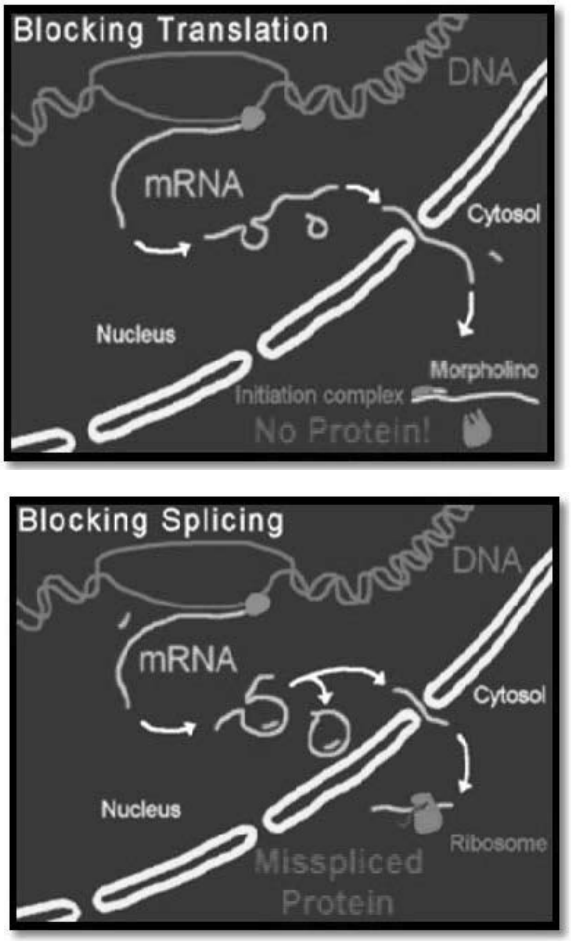

Fig. 2. *Functions of MO – A) blocking translation and B) blocking splicing (http://www.gene-tools.com/node/17#Presentations).*

1.6. Induction of germ-line chimerism

Germ cell transplantation to induce chimerism in fish is an expanding focus of biotechnology. Generally the term chimera is for an individual whose body is composed of cells from different individuals, or even different species. After transplantation of donor germ cells into a host, the transplanted viable cells migrate and colonize the genital ridge of the recipient. Later, they proliferate and differentiate into developing and then mature gametes, and may subsequently produce offspring of the donor. This biotechnology can enhance the production of gametes of species that are endangered, commercially valuable or have difficult reproduction, such as late sexual maturation, using common species as the surrogate host, one that is easily adapted to artificial reproduction (Okutsu et al., 2006b; Yamaha et al., 2007). This technique provides important advantages such as (1) shortening the reproduction cycle by using species with a shorter generation interval as hosts; (2) reducing the space required for culture when small fish species are used as hosts; (3) conservation of germ cells for maintaining genetic resource in liquid nitrogen (4) retaining target species without keeping adult fish (Yamaha et al., 2007; Yazawa et al., 2013).

This surrogate broodstock technology has already been demonstrated for model teleost species such as zebrafish goldfish or loach by single PGC transplantation (Saito et al., 2008), as well as for several species of *Salmonidae* (Takeuchi et al., 2004; Okutsu et al., 2006a, 2007). Application of this technique in *Salmonids* showed that xenogeneic transplantation of rainbow trout SG into the peritoneal cavity of sterile triploid masu salmon hatchlings, developed surrogate salmon parents which produced only donor-derived trout sperm and eggs (Okutsu et al., 2007). Another example is that of Yazawa et al. (2013) who has focused on marine aquaculture species. Their ultimate goal was to establish a small-bodied surrogate broodstock that was closely related to Pacific bluefin tuna (sexually mature in 7–8 years and weighing about 300 kg), such as mackerel *Scomber scombrus* (sexually mature in 6 months weighting 0.3 kg), to produce functional tuna gametes by spermatogonial transplantation. If this technique were to be successful, it would be possible to produce tuna seed stocks in small, land-based tanks with short generation times. Therefore the main goal of our future study is to establish a germ-line chimera within cyprinids and also within sturgeons.

First, we would like to apply this biotechnology to a common species of commercially valuable fish, such as tench, by creating a germ-line chimera with smaller and faster-reproducing fish species. We believe that the long generation interval of tench (2–3 years) might be shorten at least four times by using minnow as a surrogate host (reproductive cycle: 4–6 months).

Second, we would like to develop these techniques within sturgeons. The idea is to use sterlet, a common sturgeon species with a relatively short period of maturation and generation interval (sexually mature in 5 years) and with a smaller body size, as the host to produce gametes of a donor, for example from a critically endangered species with long reproductive cycle and large body size such as beluga (maturating in 20 years) (Fig. 3). This could result in obtaining sperm and eggs of beluga at least four times faster. The second part will be more difficult in comparison with cyprinids project because, so far little is known about germ-line chimerism in sturgeon species.

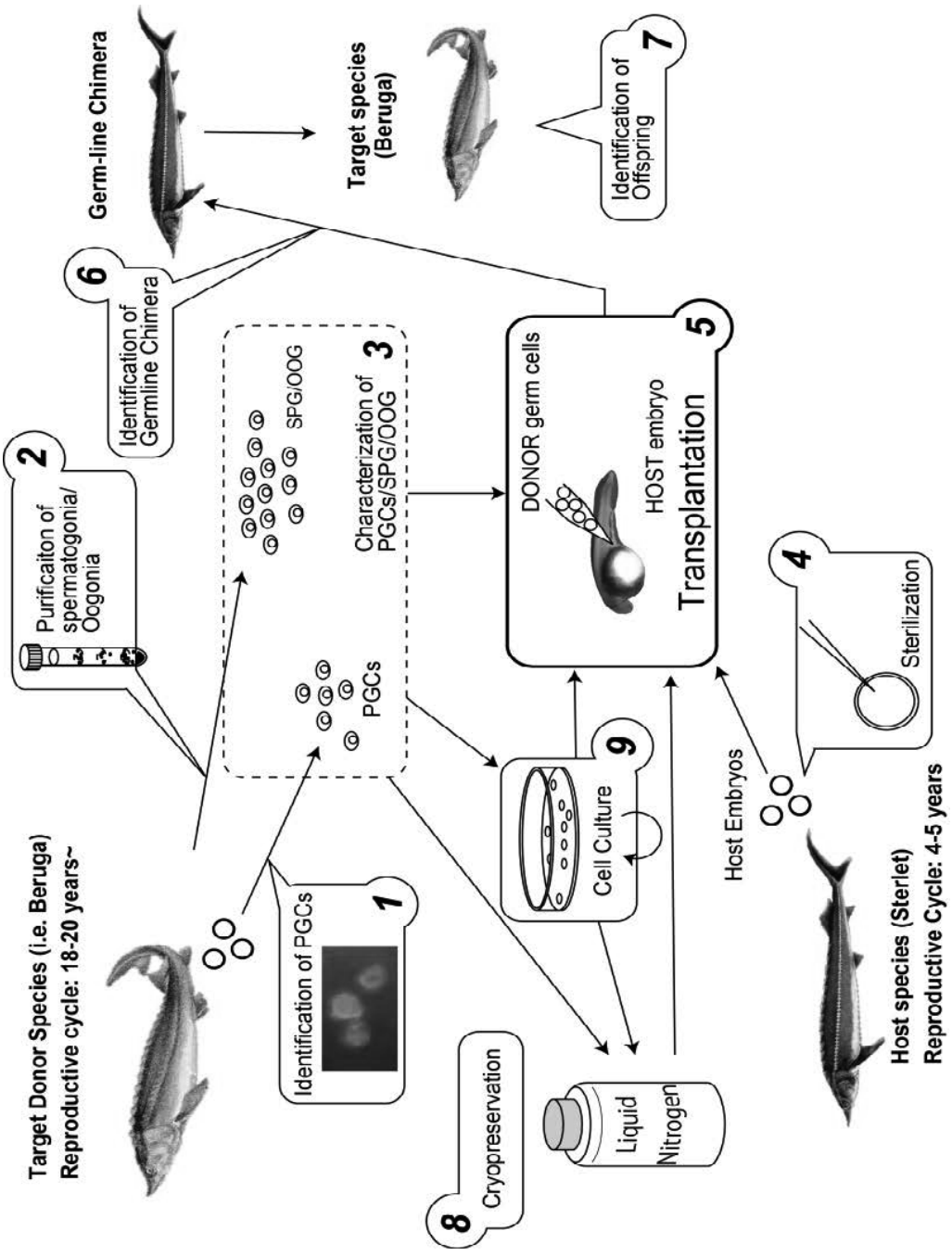

Fig. 3. *Schematic diagram for induction of germ-line chimera by transplantation of sterlet germ cells into beluga.*

1.7. Aims of the thesis

The main aims of this study were:

1) To document the embryogenesis and larval development of selected fish species, along with the origin and migration routes of germ cell lineage.
2) To develop a practical technique for isolation and cryopreservation of early stages of germ cells of selected fish.
3) To identify germ cell responsible genes and develop an efficient method of sturgeon sterilization.
4) To investigate germ-line chimerism within fish.

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CHAPTER 2

MORPHOLOGY AND ULTRASTRUCTURE OF BELUGA (*HUSO HUSO*) SPERMATOZOA AND A COMPARISON WITH RELATED STURGEONS

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Morphology and ultrastructure of beluga (*Huso huso*) spermatozoa and a comparison with related sturgeons

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ABSTRACT

The ultrastructure and morphology of beluga spermatozoa (*Huso huso*) (Acipenseridae, Chondrostei) were studied by scanning and transmission electron microscopy. Spermatozoa of 51.27 ± 4.71 μm total length (mean ± SD) were typical of sturgeon and paddlefish spermatozoa. Each consisted of an elongated nucleus (length: 5.84 ± 0.46 μm) with distinct acrosome, a cylindrical midpiece (length: 2.10 ± 0.42 μm), and a single flagellum (length: 42.21 ± 3.82 μm). The acrosome was umbrella shaped (length: 1.12 ± 0.14 μm, width: 0.87 ± 0.10 μm) with seven to nine posterolateral projections (length: 0.49 ± 0.09 μm). Three endonuclear canals, spirally arranged, traversed the nucleus length from the acrosome to the implantation fossa. The flagellum comprised an axoneme with the typical 9+2 organization of microtubules. Two flat fins were present along the flagellum parallel to the plane of the central doublet of microtubules. These fins arose at different positions, 0.57 ± 0.30 and 4.06 ± 1.32 μm from the midpiece and terminated 4.18 ± 1.09 and 4.92 ± 1.34 μm from the distal end of the flagellum. Principal component analysis revealed spermatozoan ultrastructure and morphology were similar among sturgeon species, and, although assigned to genus *Huso*, beluga may be closely related to the genus *Acipenser*.

1. Introduction

Sturgeon spermatozoa show specific differences from those of *Teleostei*, in functionality, phylogeny, and morphology (Jamieson, 1991; Mattei, 1991). The study of spermatozoa structure provides important information, not only for evaluation of sperm quality to optimize artificial reproduction, but to increase knowledge of evolutionary and taxonomic relationships among family, genera, and species (Jamieson, 1991; Mattei, 1991; Lahnsteiner and Patzner, 2008). Chondrostean spermatozoa differ fundamentally from those of teleosts in possessing an acrosome (Lahnsteiner et al., 2004; Psenicka et al., 2007), having a longer motility period (Alavi et al., 2004), and containing acrosin (Piros et al., 2002). The sturgeon spermatozoon is composed of three main parts: (1) an elongated head with an acrosome and subacrosome; (2) a cylindrical midpiece with centriolar complex, mitochondria and implantation fossa; and (3) a flagellum. The head usually contains three endonuclear canals, and the acrosome often has several posterolateral projections. The flagellum consists of an axoneme composed of nine doublets of peripheral microtubules and one central pair of singlet microtubules typical of most fish spermatozoa. The plasma membrane forms two lateral fins along most of the length of the flagellum (DiLauro et al., 2001; Psenicka et al., 2007, 2009; Wei et al., 2007; Hatef et al., 2011). The length of the fins usually differs, with one fin originating anterior to the other (Psenicka et al., 2007, present study).

Beluga, *Huso huso*, belongs to the largest fresh water chondrostean fish family, the Acipenseridae (containing 27 species), which appeared about 200 million years ago BP

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(Bemis et al., 1997; Ludwig, 2008). In the current classification, the Acipenseridae is divided into 2 subfamilies: Scaphirhynchinae and Acipenserinae, which includes the genera *Acipenser* and *Huso* (Berg, 1940; Ludwig, 2008). These fish inhabit the northern hemisphere (Bemis et al., 1997) and migrate primarily for reproduction and feeding depending on species (Billard and Lecointre, 2001). Sturgeon are currently generating interest, as most belong to endangered or critically endangered species and are listed in The International Union of Conservation of Nature Red List of Threatened Species mostly due to overfishing for caviar, poaching, environmental pollution, and late maturation and reproduction (Havelka et al., 2011). There is a suggestion that beluga should not be placed in the genus *Huso* because this genus is monophyletic (Birstein and DeSalle, 1998; Zhang et al., 2000; Ludwig et al., 2001). Birstein and DeSalle (1998) placed the two species of the genus *Huso* (beluga and Kaluga, *Huso dauricius*) in the genus *Acipenser*. They demonstrated that these species are genetically similar to those of the genus *Acipenser* and suggested a sister-species relationship for *Huso huso* and *Acipenser ruthenus*. Zhang et al. (2000) and Ludwig et al. (2000, 2001) agree that *Huso* belongs in *Acipenser*, and did not find the two species of *Huso* to be closely related.

The major aim of this study was to investigate the ultrastructure and morphology of beluga spermatozoa by using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and to compare them with spermatozoa of other sturgeon species. Nothing has been previously published on beluga spermatozoa morphology.

2. Materials and methods

The study was performed at Research Institute of Fish Culture and Hydrobiology in Vodnany, Czech Republic. Sperm was collected from broodfish at Fischzucht Rhönforelle GmbH & Co. KG, Marjoss, Germany in April 2012. Electron microscopy studies were carried out at the Laboratory of Electron Microscopy of the Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic in Ceske Budejovice.

2.1. Broodfish and sperm collection

Four *H. huso* males, 17 years of age and 40 kg average body weight, were transferred from ponds into broodfish tanks in the hatchery 5 days prior to spawning induction in April 2012. Temperature of the water in tanks was maintained at 13–15 °C. Spermiation was stimulated by a single intramuscular injection of carp pituitary powder dissolved in 0.9% NaCl at 4 mg kg^{-1} body weight. Sperm was collected 48 h after hormone injection into 50 mL cell culture containers and kept on ice at 4 °C until the samples were fixed. Spermatozoa motility was assessed by light microscopy and reached >90%.

2.2. Sample preparation

Samples were prepared according to Psenicka et al. (2007). Spermatozoa were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and stored for 48 h at 4 °C. Samples were washed in 0.1 M phosphate buffer + 4% glucose and post-fixed in 4% osmium tetroxide in 0.1 M phosphate buffer (1:1) for 2 h at room temperature. For SEM, a drop of sperm was placed on a circular coverslip coated with polylysine and held for 2 h at room temperature. The remainder of each sample was used for TEM, where sperm was transferred into 2% agar heated to 62 °C, immediately centrifuged for 10 min at 510 × *g* and cooled for 30 min at 4 °C to solidify. Samples for both studies were dehydrated through an acetone series (30, 50, 70, 90, 95, and 100% acetone for 15 min). Samples for SEM were completely dried with a Pelco CPD2 critical point dryer (Ted Pella, Inc., Redding, CA) attached to a metal stubs with carbon tape and coated with gold under vacuum using SEM Coating Unit E5100 (Polaron Equipment Ltd., England). The samples were examined with a scanning electron microscope JSM-7401F (JEOL Ltd., Tokyo, Japan). For TEM, the samples were incubated in 3 mixtures of 100% acetone and resin (Epon 812, Polysciences, Inc.) of increasing resin ratios (2:1, 1:1, 1:2) for 1 h to replace acetone and finally placed in 100% resin in a desiccator connected to a water vacuum pump for 24 h to improve the impregnation of specimens with the resin. After 24 h, samples were embedded in the new pure resin and polymerized for 48 h in an incubator at 62 °C, then cut into ultrathin sections of 50–70 nm using a Leica UCT ultramicrotome (Leica Microsystems, Germany). Sections were double-stained with a saturated solution of uranyl acetate in 50% ethanol and lead citrate according Venable and Coggeshall (1965) and washed 3 times in 30% ethanol between the first and second staining and with distilled water after the second staining. The prepared samples were examined with a JEM 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at 80 kV. Morphological parameters of spermatozoa were measured from recorded pictures using Olympus MicroImage software (version 4.0.1. for Windows, Germany).

2.3. Data analysis

We measured the following spermatozoon features for each male from SEM micrographs: acrosome length (AL) and width (AW), nucleus length (NL), anterior nucleus width (ANW), central nucleus width (CNW), posterior nucleus width (PNW), midpiece length (ML), anterior midpiece width (AMW), posterior midpiece width (PMW), flagellum length (FL), and total length (TL) (Table 1). Spermatozoon features measured from photographs obtained by TEM were: endonuclear canal diameter (ECD), flagellum diameter (FD), flagellum fin width (FW), peripheral doublets axoneme microtubules width (PDMW), central doublet axoneme microtubules width (CDMW), diameter of peripheral single microtubule (PDM), diameter of single central microtubule (CDM). Means ± standard deviations (SD) were calculated for each male (Table 2). Differences were assessed by ANOVA with post hoc comparisons using Tukey's HSD test. Probability values of $P < 0.05$ were considered significant. A principal component analysis (PCA) was used to identify the relationships among the most important size characteristics of the beluga spermatozoon and also of those reported for spermatozoa of four other sturgeon species [Siberian, *Acipenser baerii* (Psenicka et al.,

Table 1
Biometric measurements of beluga spermatozoa made on SEM photographs from 4 fish.

Biometric character	Measurement (μm) for each fish				
	1	2	3	4	Mean
TL	$52.21^{b,c} \pm 4.86$ [23] (42.14–65.23)	$53.98^{c} \pm 3.78$ [23] (50.03–66.02)	$50.06^{a,b} \pm 4.40$ [25] (43.56–61.42)	$48.83^{a} \pm 3.01$ [28] (44.50–58.54)	51.27 ± 4.71 [99] (42.14–66.02)
NL	$5.77^{a} \pm 0.49$ [23] (4.11–6.43)	$6.37^{b} \pm 0.43$ [23] (5.46–7.05)	$5.47^{a} \pm 0.30$ [25] (4.93–6.03)	$5.74^{a} \pm 0.63$ [28] (3.73–6.66)	5.84 ± 0.46 [99] (4.11–7.05)
AL	$1.13^{b} \pm 0.17$ [23] (0.87–1.49)	$0.98^{a} \pm 0.12$ [23] (0.79–1.19)	$1.15^{b} \pm 0.15$ [25] (0.88–1.58)	$0.96^{a} \pm 0.13$ [28] (0.60–1.22)	1.12 ± 0.14 [99] (0.60–1.58)
AW	$0.91^{a} \pm 0.13$ [23] (0.71–1.40)	$0.91^{a} \pm 0.12$ [23] (0.70–1.15)	$0.87^{ab} \pm 0.06$ [25] (0.77–1.03)	$0.80^{b} \pm 0.09$ [28] (0.64–0.98)	0.87 ± 0.10 [99] (0.64–1.40)
ANW	$0.69^{a} \pm 0.04$ [23] (0.61–0.78)	$0.70^{a} \pm 0.08$ [23] (0.58–0.90)	$0.69^{a} \pm 0.05$ [25] (0.60–0.85)	$0.68^{a} \pm 0.08$ [28] (0.57–1.01)	0.69 ± 0.06 [99] (0.57–1.01)
CNW	$0.91^{ab} \pm 0.05$ [23] (0.81–1.02)	$0.90^{a} \pm 0.08$ [23] (0.71–1.11)	$0.94^{b} \pm 0.05$ [25] (0.86–1.09)	$0.90^{a} \pm 0.05$ [28] (0.81–1.04)	0.91 ± 0.06 [99] (0.71–1.11)
PNW	$0.99^{a} \pm 0.07$ [23] (0.84–1.12)	$0.97^{a} \pm 0.08$ [23] (0.84–1.17)	$1.00^{a} \pm 0.06$ [25] (0.84–1.17)	$0.95^{a} \pm 0.09$ [28] (0.78–1.25)	0.98 ± 0.08 [99] (0.78–1.25)
ML	$1.95^{a} \pm 0.30$ [23] (1.37–2.43)	$2.07^{a} \pm 0.58$ [23] (0.64–2.89)	$2.00^{a} \pm 0.30$ [25] (1.42–2.68)	$1.76^{a} \pm 0.50$ [28] (0.64–2.77)	2.10 ± 0.42 [99] (0.64–2.89)
AMW	$0.65^{a} \pm 0.09$ [23] (0.46–0.91)	$0.59^{a} \pm 0.09$ [23] (0.38–0.75)	$0.64^{a} \pm 0.07$ [25] (0.46–0.75)	$0.62^{a} \pm 0.08$ [28] (0.46–0.78)	0.63 ± 0.08 [99] (0.38–0.91)
PMW	$0.57^{a} \pm 0.07$ [23] (0.46–0.91)	$0.63^{a} \pm 0.11$ [23] (0.40–0.91)	$0.56^{a} \pm 0.08$ [25] (0.37–0.68)	$0.57^{a} \pm 0.09$ [28] (0.41–0.76)	0.58 ± 0.09 [99] (0.37–0.91)
FL	$43.36^{bc} \pm 4.88$ [23] (33.03–57.04)	$44.57^{c} \pm 3.46$ [23] (40.51–55.96)	$41.47^{ab} \pm 4.18$ [25] (34.62–51.78)	$40.36^{a} \pm 2.77$ [28] (36.18–49.56)	42.21 ± 3.82 [99] (33.03–57.04)

Measurements are shown as means ± standard deviations (SD) for *n* spermatozoa (square brackets). The range of measurements is given parentheses. Values with the same letter are not significantly different (ANOVA, Tukey's HSD test, $P < 0.05$). Data are measured in μm. AL, acrosome length; AMW, anterior midpiece width; ANW, anterior nucleus width; AW, acrosome width; CNW, central nucleus width; FL, flagellum length; ML, midpiece length; NL, nucleus length without midpiece or acrosome; PMW, posterior midpiece width; PNW, posterior nucleus width; TL, total length (nucleus with acrosome, midpiece, flagellum).

2007); Russian, *Acipenser gueldenstaedti* (Hatef et al., 2012); Persian sturgeon, *Acipenser persius* (Hatef et al., 2011); and sterlet, *A. ruthenus* (Psenicka et al., 2009)], which could be used as key features for intra- and inter-species variations. All statistical analysis was performed using Statistica for Windows, Version 9.1 (StatSoft, Inc.).

3. Results

3.1. General morphological characteristics

The spermatozoon of beluga is elongated, radially symmetrical, cylindrical, and uniflagellated. It can be

Table 2
Biometric measurements of beluga spermatozoa made on TEM from 4 fish.

Biometric character	Measurement (nm) for each fish				
	1	2	3	4	Mean
FW	$657.63^{a} \pm 144.36$ [12] 469.84–881.72	$700.23^{a} \pm 161.11$ [10] 514.70–931.17	$627.43^{a} \pm 76.95$ [7] 509.92–710.73	$870.33^{b} \pm 78.60$ [5] 735.27–957.49	713.90 ± 92.20 [34] 469.84–957.49
FD	$150.73^{a} \pm 11.69$ [14] 142.05–170.20	$154.69^{ab} \pm 8.93$ [11] 142.30–165.03	$157.02^{a} \pm 4.63$ [7] 150.35–163.80	$158.54^{a} \pm 8.82$ [8] 145.33–171.45	155.24 ± 6.82 [40] 142.05–171.45
CDMW	$43.23^{a} \pm 5.52$ [14] 36.30–53.35	$42.34^{a} \pm 2.07$ [7] 39.52–45.83	$34.84^{b} \pm 6.12$ [7] 23.78–40.49	$38.86^{ab} \pm 2.14$ [5] 36.34–42.76	39.81 ± 3.17 [30] 23.78–53.35
PDMW	$27.05^{a} \pm 2.24$ [11] 23.62–33.34	$28.43^{a} \pm 4.48$ [10] 24.44–39.79	$25.47^{a} \pm 1.47$ [8] 22.86–27.31	$25.17^{a} \pm 4.52$ [10] 12.84–29.02	26.52 ± 2.54 [47] 12.84–33.34
CDM	$13.43^{a} \pm 2.18$ [10] 10.43–16.50	$14.66^{a} \pm 2.16$ [7] 11.54–17.5	$13.02^{a} \pm 8.60$ [5] 12.19–14.66	$13.00^{a} \pm 1.55$ [5] 10.09–14.55	13.52 ± 1.35 [27] 10.09–17.5
PDM	$12.05^{ab} \pm 1.80$ [13] 8.98–15.68	$14.29^{b} \pm 1.23$ [6] 12.71–16.29	$10.91^{a} \pm 1.69$ [10] 8.57–13.66	$10.95^{a} \pm 1.86$ [8] 7.92–13.78	12.05 ± 1.32 [37] 8.57–16.29
ECD	$50.04^{a} \pm 5.44$ [42] 36.81–60.05	$48.86^{a} \pm 7.79$ [18] 37.97–69.29	$47.24^{a} \pm 5.52$ [36] 32.71–55.37	$49.46^{a} \pm 7.91$ [25] 37.89–69.48	48.90 ± 5.33 [121] 32.71–69.48

Measurements (nm) are shown as means ± standard deviations (SD) for *n* spermatozoa (square brackets). The range of measurements is given parentheses. Values with the same letter are not significantly different (ANOVA, Tukey's HSD test, $P < 0.05$). CDM, diameter of one central microtubule; CDMW, central doublet of axoneme microtubules width; FW, flagellum diameter; FW, flagellum fin width; ECD, endonuclear canal diameter; PDMW, peripheral doublet axoneme microtubules width; PDM, diameter of one peripheral microtubule.

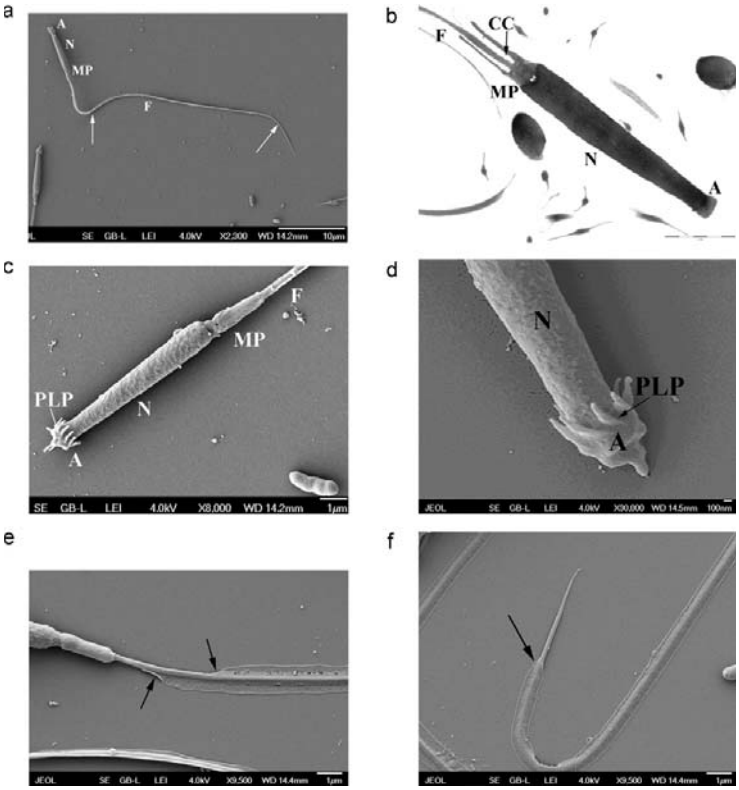

Fig. 1. General structure of beluga spermatozoon. (a, c, d and e) SEM and (b) TEM show: (a and b) all parts of the spermatozoon, scale bars 10 μm and 2 μm; (c) detail of the head, scale bar 1 μm; (d) detail of acrosome with PLP, scale bar 100 nm; (e) origin of the fins along the flagellum, scale bar 1 μm; (f) end of the fins along the flagellum, scale bar 1 μm. The arrows show development of the fins along the flagellum. Acrosome (A), cytoplasmic channel (CC), flagellum (F), midpiece (MP), nucleus (N), posterolateral projections (PLP).

differentiated into an elongated head with an acrosome, a cylindrical midpiece, and a flagellar region with fins (Figs. 1–4). The mean total length of spermatozoa among the four males (mean ± S.D. of the nucleus with acrosome, midpiece, and flagellum) was: 52.21 ± 4.86, 53.98 ± 3.78, 50.06 ± 4.40, and 48.83 ± 3.01 μm, with an overall average total length of 51.27 ± 4.71 μm (Tables 1 and 2). Principal component analysis conducted on 8 variables showed 57.49% of the total accumulated variance to be accounted for by three factors (Table 3). The first two factors (PCA 1 and PCA 2) accounted for 42.33% of the variation among individual fish (Fig. 5 and Table 3). The strength of each variable in each component showed nucleus width (NW) to have the largest positive coefficient (0.73) in factor 1, and NL had the largest negative coefficient (−0.82) in this factor, indicating that the nucleus had greatest effect in factor 1 (Table 3). The scores for the first and second component (PCA 1 and PCA 2) demonstrated that the acrosome, nucleus, midpiece, and flagellum show inter-individual variation in this species (Fig. 6). The PCA determined the relationships among measured morphological parameters as a negative dependency or independency (Fig. 5). We grouped the variables relating to nucleus width into a single parameter: the mean of anterior, central, and posterior width. A significant negative correlation was observed

Table 3
Factor loading on the components extracted by PCA of all beluga spermatozoon characters.

Morphological character	Factor		
	1	2	3
NL	−0.82	−0.15	−0.01
AL	0.42	−0.42	−0.44
AW	0.10	−0.60	0.17
NW	0.73	−0.20	0.38
ML	−0.47	−0.58	−0.38
AMW	0.38	−0.40	−0.53
PMW	−0.07	−0.49	0.57
FL	−0.20	−0.50	0.32

AL, acrosome length; AMW, anterior midpiece width; AW, acrosome width; FL, flagellum length; NL, nucleus length; ML, midpiece length; NW, nucleus width; PMW, posterior midpiece width.

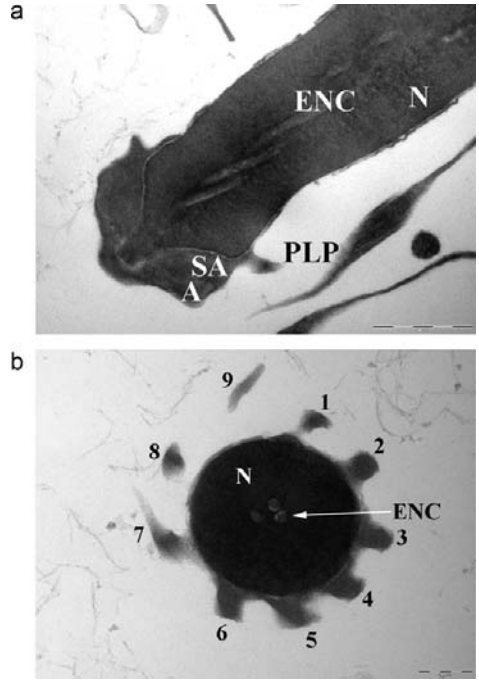

Fig. 2. Ultrastructure of acrosome of beluga spermatozoon. Longitudinal (a) and cross (b) sections show: acrosome (A), subacrosome (SA), endonuclear canals (ENC) traversing the whole length of nucleus (N) in (a), nine posterolateral projections (PLP) in (b). Scale bars are 500 nm (a) and 200 nm (b).

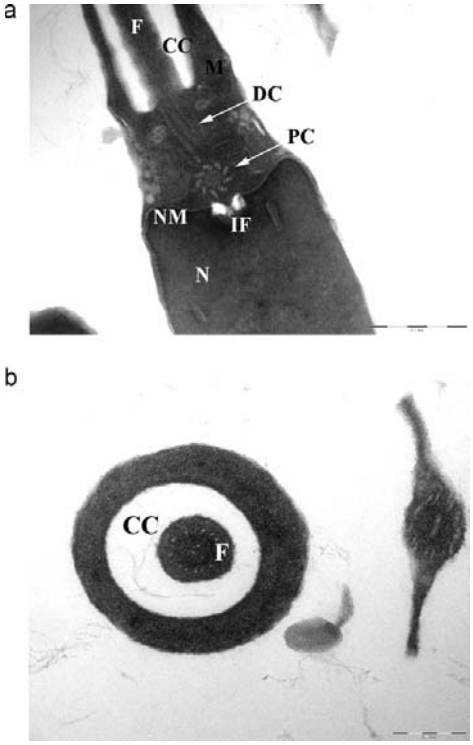

Fig. 3. Ultrastructure of midpiece of beluga spermatozoon. Longitudinal (a) and cross (b) sections show: nucleus (N) surrounded by nuclear membrane (NM), flagellum (F) separated by cytoplasmic channel (CC), mitochondrion (M), distal centriole (DC) and proximal centriole (PM) near the implantation fossa (IF). Scale bars are 500 nm (a) and 200 nm (b).

between NL and NW. Hence when NL is shorter, NW is wider and vice versa, suggesting that the volume of nuclear material must be similar in each sperm cell.

Comparison of beluga spermatozoon morphology with that of the four other sturgeon species by factor loading of the components extracted by PCA (PCA 1 and PCA 2) demonstrated that the measured characteristics of sturgeon spermatozoa show significant inter-specific variations (Fig. 7). We summarized the morphological characteristics of these species, and of seven additional sturgeon species (Table 4).

3.2. Acrosome

The acrosome situated in the anterior of the nucleus had an umbrella shape (Figs. 1a–d and 2a). Mean acrosomal length and width were 1.12 ± 0.14 μm and 0.87 ± 0.10 μm (Table 1). Longitudinal sections showed a subacrosome region between the nucleus and the acrosome, and cross-sections revealed 7–9 posterolateral projections (PLP) (Fig. 2b). The PLPs arose from the posterior edge of the acrosome and extended along the sides of the head. Mean length was 0.49 ± 0.09 μm.

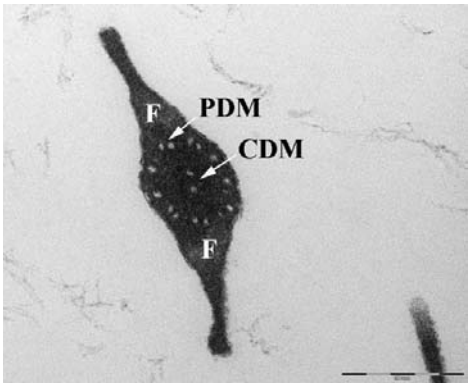

Fig. 4. Ultrastructure of flagellum of beluga spermatozoon. Cross section shows two fins (F), the axoneme with peripheral doublets of microtubules (PDM) and central doublet of microtubules (CDM). Scale bar, 200 nm.

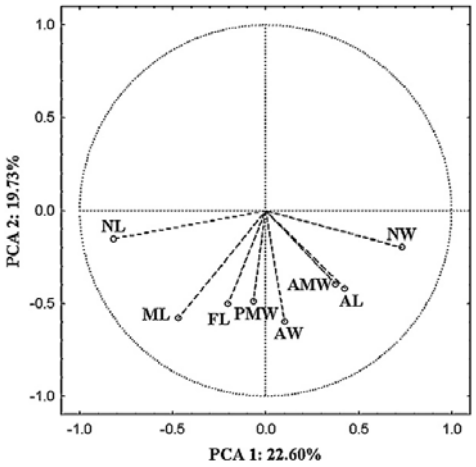

Fig. 5. PCA diagram of spermatozoon morphological parameters of beluga. AL, acrosome length; AMW, anterior midpiece width; AW, acrosome width; FL, flagellum length; NL, nucleus length; ML, midpiece length; NW, nucleus width; PMW, posterior midpiece width.

3.3. Head

The head had an elongated trapezoid shape. Mean length without acrosome was 5.84 ± 0.46 μm. The nucleus occupied most of the head and was composed of electron-dense and granular materials (chromatin) surrounded by a nuclear membrane. Mean NW at three points: anterior (proximal to the acrosome), central (center of the head), and posterior (near the midpiece) was 0.69 ± 0.06, 0.91 ± 0.06, and 0.98 ± 0.08 μm, respectively (Table 1). Three ENCs of similar size were observed, mean diameter 48.90 ± 5.33 nm. The ENCs appeared as spiral structures that traversed the nucleus from the junction with the acrosome to the basal nuclear fossa region (Fig. 2a and b). The organelle, implantation fossa, connecting the axial portion of the nucleus with the midpiece, was located at the posterior of the nucleus (Fig. 3a).

3.4. Midpiece

The midpiece is cylindrical, elongated at its posterior, and contains mitochondria and a centriolar apparatus. The mean midpiece length was 2.10 ± 0.42 μm, tapering from anterior (0.63 ± 0.08 μm) to posterior (0.58 ± 0.09 μm). Several mitochondria (3–6) were observed in the peripheral area. The distal centriole acts as the flagellar basal body, and the proximal centriole was near the implantation fossa. Both centrioles had a cylindrical shape and were composed of nine peripheral triplets of microtubules (Fig. 3a). The flagellum was surrounded by a cytoplasmic channel formed by an invagination at the plasmalemma. This channel separated the flagellum from the midpiece by an extracellular space (Fig. 3b).

3.5. Flagellum

Overall mean FL was 42.21 ± 3.82 μm. Flagella contained an internal axoneme with typical eukaryotic organization: one central pair of single microtubules surrounded by nine peripheral doublets of microtubules (Fig. 4). The mean diameters of the central and peripheral microtubules were 13.52 ± 1.35 and 12.05 ± 1.32 nm, respectively. The mean diameter of the central pair and the peripheral doublet microtubules were 39.81 ± 3.17 and 26.52 ± 2.54 nm, respectively (Table 2). The diameter of the flagellum was measured orthogonally in a straight line connecting the central microtubules and was 155.24 ± 6.82 nm. We observed two independent lateral extensions of the flagellar membrane, the fins, arising in the vicinity of the midpiece and extending to the distal region of the flagellum. The fins were flat with no twist and co-planar with the plane bisecting the central doublet of

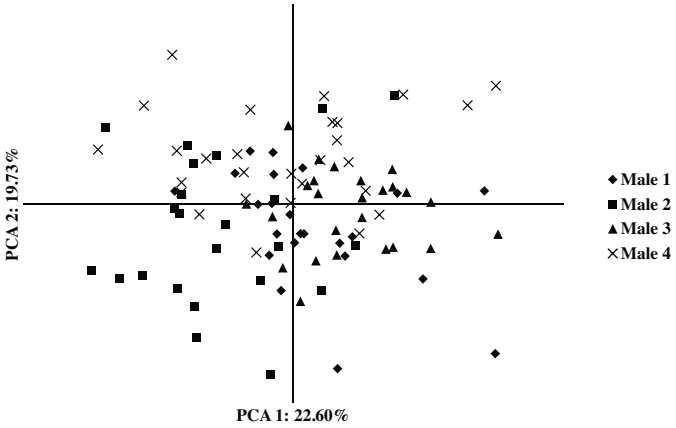

Fig. 6. Individual variations in spermatozoon morphology of four males of beluga using scores for the first and second principal component in a PCA.

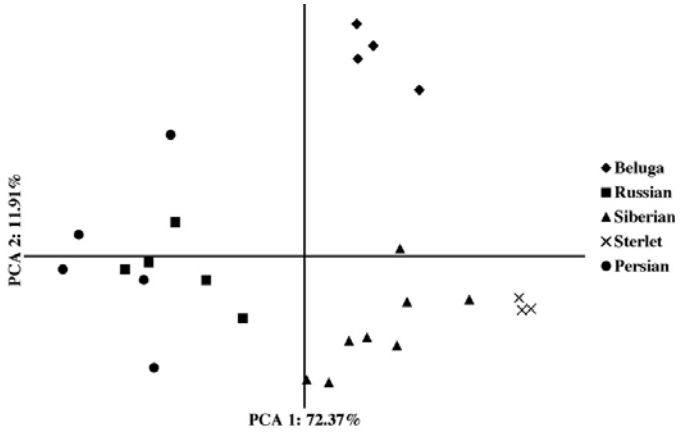

Fig. 7. Individual variations in spermatozoon morphology of 5 sturgeon species using scores for the first and second principal component in a PCA.

microtubules of the axoneme. The position of the origin of the fins consistently differed, but nearly always terminated at the same point (Fig. 1e and f). The fins arose, on average, 0.57 ± 0.30 and 4.06 ± 1.32 μm from the midpiece and terminated 4.18 ± 1.09 and 4.92 ± 1.34 μm, respectively, from the end of flagellum.

4. Discussion

4.1. General morphological characteristics

The present study showed the ultrastructure and morphology of beluga spermatozoa to be similar to that of other sturgeon species. These include Russian sturgeon, stellate sturgeon *Acipenser stellatus*, white sturgeon *Acipenser transmontanus*, Atlantic sturgeon *Acipenser oxyrhynchus*, shortnose sturgeon *Acipenser brevirostrum*, lake sturgeon *Acipenser fulvescens*, pallid sturgeon *Scaphirhynchus albus*, paddlefish *Polyodon spathula* (Zarnescu, 2005), Siberian sturgeon, Chinese sturgeon *Acipenser sinensis*, sterlet, and Persian sturgeon (Table 4).

As commonly seen in members of the Acipenseridae, beluga spermatozoa can be differentiated into an elongated nucleus with an acrosome, a cylindrical midpiece, and a flagellum with two fins extending almost the entire length, with typical axoneme structure of 9+2 organization of microtubules. These results have confirmed the suggestion (Jamieson, 1991; DiLauro et al., 2001; Psenicka et al., 2007) that the morphology and ultrastructure of sturgeon spermatozoa is of taxonomic value and can be a basis for defining phylogenic relationships among these species. Total length of beluga spermatozoa is similar to that of Siberian sturgeon (Table 4).

Our results can be seen as support for the suggestion of many researchers that beluga is closely related to *Acipenser* and might not belong in *Huso* (Birstein and DeSalle, 1998; Zhang et al., 2000; Ludwig et al., 2001). Since the spermatozoon ultrastructure of Kaluga, the other species of *Huso*, has not been described, it was not compared with that of beluga. Our results support the inclusion of this species within sturgeons. Beluga occurs primarily in the Atlantic, and Kaluga is clustered with the green sturgeon, *Acipenser medirostris*, and Sakhalin sturgeon, *Acipenser mikadoi*, in the Pacific (Krieger et al., 2008). The two species also differ in the number of chromosomes. Beluga (116 ± 4 chromosomes) (Fontana and Colombo, 1974) and sterlet (118 ± 2 chromosomes) (Fontana et al., 1975) are considered to be modern (functional) diploids, while Kaluga (268 ± 4 chromosomes) (Vasil'ev et al., 2009) is considered tetraploid (Havelka et al., 2011). The assignment of beluga and Kaluga to the *Huso* genus is mainly due to morphology of this species and historical usage. Knowledge of taxonomic and phylogenetic relationships of sturgeon is still limited to their morphological variability and ability to produce fertile hybrids between taxonomically distant species (Havelka et al., 2011).

4.2. Acrosome

Most spermatozoa of teleost fishes lack an acrosome, which is compensated for by the presence of a micropyle on the egg surface through which penetration of the spermatozoon occurs (Lahnsteiner and Patzner, 1999). The spermatozoa of sturgeon and paddlefish possess an acrosome, and their eggs have several micropyles (Linhart and Kudo, 1997; Debus et al., 2008; Psenicka et al., 2010a). The acrosome is considered to be derived from the Golgi apparatus (Nicander, 1970). Psenicka et al. (2010a) proposed the function of the acrosome with PLPs and a fertilization filament as an anchor and a spear, respectively, to improve penetration of the egg in fast-flowing waters. The present study showed that the AL and AW of the beluga are most similar to those of Persian, pallid, stellate, and Siberian sturgeon, with slight differences in their dimensions.

The number and size of PLPs vary among species (DiLauro et al., 2001; Psenicka et al., 2007; Wei et al., 2007).

Table 4
Biometric characteristics of spermatozoa in various sturgeon species.

Species	AL	AW	NL	ANW	PNW	ML	MW	FL	TL	*n*	References
Beluga	1.12 (0.14)	0.87 (0.10)	5.84 (0.46)	0.69 (0.06)	0.98 (0.08)	2.10 (0.42)	0.61 (0.09)	42.21 (3.82)	51.27 (4.71)	4	Present study
Russian sturgeon	1.18 (0.01)	1.05 (0.01)	6.84 (0.04)	1.10 (0.01)	1.48 (0.01)	1.64 (0.03)	0.92 (0.01) (A) 0.62 (0.01) (P)	49.42 (0.37)	57.08 (0.79)	5	Hatef et al. (2012)
Persian sturgeon	1.15 (0.15)	1.16 (0.15)	7.05 (0.51)	1.24 (0.15)	1.54 (0.17)	1.81 (0.46)	0.90 (0.12) (A) 0.73 (0.13) (P)	50.31 (5.87)	59.18 (6.23)	5	Hatef et al. (2011)
Sterlet	0.79 (0.07)	0.75 (0.07)	3.30 (0.31)	0.67 (0.07)	0.85 (0.08)	0.97 (0.23)	0.64 (0.12) (A) 0.48 (0.11) (P)	42.47 (1.89)	47.61 (1.89)	3	Psenicka et al. (2008, 2009)
Chinese sturgeon	0.54 (0.15)	0.68 (0.06)		0.59 (0.05)	1.84 (0.45)	2.17 (0.36)	1.57 (0.27)	33.26 (2.74)	38.7		Wei et al. (2007)
Siberian sturgeon	0.95 (0.17)	0.93 (0.12)	4.98 (0.83)	0.87 (0.13)	1.14 (0.18)	1.09 (0.43)	0.80 (0.25) (A) 0.57 (0.19) (P)	44.75 (4.93)	51.76	8	Psenicka et al. (2007)
Pallid sturgeon	1.07 (0.10)	0.82 (0.06)	3.78 (0.33)	0.68 (0.04)	0.89 (0.06)	1.23 (0.16)	0.67 (0.08)	37.16	43.23	16	DiLauro et al. (2001)
Lake sturgeon	0.73 (0.14)	0.81 (0.07)	5.69 (0.43)	0.68 (0.07)	1.04 (0.08)	2.68 (0.43)	0.70 (0.08)	47.53	56.63	14	DiLauro et al. (2000)
Shortnose sturgeon	0.78 (0.08)	0.91 (0.06)	6.99 (0.83)	0.75 (0.11)	1.21 (0.12)	1.91 (0.35)	0.81 (0.09)	36.7	46.41	15	DiLauro et al. (1999)
Atlantic sturgeon	0.83 (0.11)	1.00 (0.07)	3.15 (0.36)	0.92 (0.06)	0.55 (0.08)	1.37 (0.16)	0.51 (0.07)	37.08	42.74	12	DiLauro et al. (1998)
White sturgeon	1.31	1.34	9.21	1.25	1.44	2.13	1.08	30–40	41.82–51.82	1	Cherr and Clark (1984)
Stellate sturgeon	0.97	1.22	6.66	0.98	1.49	3.43	1.38	40–70	51.05–81.05	1	Ginzburg (1977)

Measurements (μm) are means ± SD (in parentheses) for *n* spermatozoa. A, anterior; AL, acrosome length; ANW, anterior nucleus width; AW, acrosome width; FL, flagellum length; ML, midpiece length; MW, midpiece width; NL, nucleus length; P, posterior; PNW, posterior nucleus width; TL, total length (nucleus with acrosome, midpiece and flagella).

We observed seven to nine in beluga, while Persian sturgeon (Hatef et al., 2011) and sterlet have 9–10 (Psenicka et al., 2009), Chinese (Wei et al., 2007) and Siberian sturgeon (Psenicka et al., 2007) have 10, pallid sturgeon has 8 (DiLauro et al., 2001), and stellate sturgeon (Dettlaff et al., 1993) have 12 PLPs. The length of PLPs in beluga spermatozoa (0.49 ± 0.09 μm) is close to that in Siberian (0.60 μm) (Psenicka et al., 2007) and pallid (0.76 μm) (DiLauro et al., 2001) sturgeon. The length of PLPs in lake (DiLauro et al., 2000), Atlantic (DiLauro et al., 1998), shortnose (DiLauro et al., 1999), Chinese (Wei et al., 2007) sturgeon, and sterlet (Psenicka et al., 2009) has been reported to be 0.32, 0.23, 0.25, 0.37, and 0.30 μm, respectively.

4.3. Head

Differences in head shape and size are evident among species. The NL (head without acrosome) of beluga spermatozoa (5.84 ± 0.46 μm) is comparable to that in lake sturgeon (5.69 ± 0.43 μm) and is considered medium-long with respect to other sturgeon spermatozoa. Shorter nuclei have been reported in Atlantic sturgeon (3.15 ± 0.36 μm) (DiLauro et al., 1998), pallid sturgeon (3.78 ± 0.33 μm) (DiLauro et al., 2001), and sterlet (3.30 ± 0.31 μm) (Psenicka et al., 2009), and longer in stellate (6.66 μm) (Dettlaff et al., 1993), shortnose (6.99 ± 0.83 μm) (DiLauro et al., 1999), Persian (7.05 ± 0.51 μm) (Hatef et al., 2011), and white sturgeon (9.21 μm) (Cherr and Clark, 1984). Inter-specific diversity in size and shape of sturgeon nuclei has evolutionary and genetic implications (DiLauro et al., 1999). The smaller nucleus allows tightly packed chromatin and nucleic acids (DiLauro et al., 1998, 2001). Endonuclear canals have been described in spermatozoa of many sturgeon species (Ginzburg, 1977; Cherr and Clark, 1984; DiLauro et al., 1998, 2000, 2001; Psenicka et al., 2007, 2008, 2009; Hatef et al., 2011). As with beluga in the present study, sturgeon generally possesses three ENCs in the nucleus, although Atlantic sturgeon have two (DiLauro et al., 1998) and Siberian sturgeon may rarely show two or four ENCs (Psenicka et al., 2008). The diameter of ENCs differs among sturgeon species and have been measured in Atlantic, shortnose, lake, pallid, Siberian, and Chinese sturgeon and sterlet as 35, 97, 49, 57.41, 44.59, 80.00, and 40.13 ± 4.61 nm, respectively (Psenicka et al., 2009). The average diameter of ENCs of beluga spermatozoa (48.90 ± 5.33 nm) was similar to those of lake sturgeon. The ENCs serve for communication of the acrosome with the implantation fossa (Psenicka et al., 2009). DiLauro et al. (1999) suggested that ENCs assist in the transfer of the centriole into the egg during fertilization. We observed an implantation fossa at the posterior end of the nucleus that was similar to that in other sturgeon species.

4.4. Midpiece

The midpiece of the beluga spermatozoon was elongated and nearly radially symmetry. The length of the beluga midpiece (2.10 ± 0.42 μm) was comparable to that in Chinese sturgeon spermatozoa (2.17 ± 0.36). The midpiece contains mitochondria and proximal and distal centrioles. Mitochondria play an important role in sperm

motility, as the main source for generation of ATP used by the axoneme (Billard et al., 1999). More studies are needed to investigate the possible correlation between the number of mitochondria, ATP production/consumption, and flagellar beat characteristics. Both proximal and distal centrioles have been observed in the midpiece of beluga sperm. Similar to all sturgeon species, the proximal centriole of the beluga spermatozoon is attached to implantation fossa by several thin strands located in the center of the nuclear fossa (Psenicka et al., 2007), and the distal centriole is located at the base of the flagellum (Psenicka et al., 2009). Data from previous studies show that centriole size differs inter- as well as intra-specifically (Psenicka et al., 2009).

4.5. Flagellum

As in other fish, the flagellum of beluga spermatozoa is separated from the midpiece by the cytoplasmatic channel, and consists of the typical axoneme with 9+2 arrangement of microtubules. The flagellar length (Table 4) and size of flagellar fins appeared to differ among sturgeon species. The flagellar length of beluga (42.21 ± 3.82 μm) was similar to that of sterlet flagellum (42.47 ± 1.89 μm). We observed two fins along the flagellum similar to those in pallid (DiLauro et al., 2001), Chinese (Wei et al., 2007), Siberian (Psenicka et al., 2007), and Persian sturgeon (Hatef et al., 2011) and sterlet (Psenicka et al., 2009), with one fin arising anterior to the other, a feature typical of sturgeon, while the fins terminated almost at the same position (4.18 ± 1.09 and 4.92 ± 1.34 μm from the end of flagella), which has not been previously reported in sturgeon species (Fig. 1e). The suggested role of these fins is to enhance the force of flagellar movement (Psenicka et al., 2008), and the motility and velocity in species with flagellar fins is significantly higher than species lacking fins (Alavi et al., 2009). The role of fins in improving hydrodynamic efficiency of sturgeon flagella was confirmed by recent results (Gillies et al., 2012).

An objective of the study was to describe the morphology and ultrastructure of beluga spermatozoa and to compare our results with that of other sturgeon species. It could be argued that results of other studies may not be comparable, due to different methods of sample processing. Psenicka et al. (2010b) studied the dimensions of sterlet spermatozoa using different electron microscopic techniques and found significant differences (almost 30%) between methods. Nevertheless we were confident about comparing data with others (Fig. 7), because most (Psenicka et al., 2007, 2008, 2009, 2010a,b; Hatef et al., 2011, 2012) were performed by our team using the same methodology.

5. Conclusion

Sturgeons possess "acrosomal aquasperm" which differ from that of teleosts, with possess spermatozoa lacking an acrosome. The present study suggested variations in morphology and ultrastructure of spermatozoa among sturgeon species. Inter-species comparisons show beluga to possess a relatively long sperm cell similar to that of Siberian sturgeon, although it more closely resembles lake sturgeon in NL and sterlet in FL. Unusual for sturgeon spermatozoa is the termination of flagellar fins almost at the same position and a variance in number of PLPs (7–9) found in beluga. The similarity in spermatozoa morphology, along with the close genetic relationship, supports the argument of Birstein and DeSalle (1998) to include beluga, presently assigned to the genus *Huso*, in the genus *Acipenser*. However, the morphology and ultrastructure of spermatozoa of Kaluga has not been reported, but should be a subject for future research to determine its relationship with beluga and other sturgeon.

Acknowledgments

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Chapter 3

EMBRYOGENESIS, VISUALIZATION AND MIGRATION OF PRIMORDIAL GERM CELLS IN TENCH (*TINCA TINCA*)

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Embryogenesis, visualization and migration of primordial germ cells in tench (*Tinca tinca*)

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Summary

Embryonic and larval development of the tench, *Tinca tinca*, is described, along with the origin and migration routes of germ cell lineage. Primordial germ cells (PGCs) potentially transmit genetic information to the next generation, and represent a powerful tool for creating a germ-line chimera within fish species. PGCs were identified by injecting synthesized mRNA, combining green fluorescent protein and the zebrafish *nos*1 3′UTR, under the blastodisc of embryos at the 1–4 cell stage. Developmental stages were divided into five periods defined by morphological features: cleavage (40 min–5 h post-fertilization (hpf), blastula (3.6–12 hpf), gastrula (12–20 hpf), segmentation (20–65 hpf), and hatching (65–188 hpf). The migration pathways of fluorescent PGCs were detected from 100% epiboly (18 hpf) to the end of the hatching period (184 hpf) in 69.3% of injected embryos, which migrated to the site of future gonads. Each hatching larva possessed 3–10 labeled PGCs with 95.1% of these cells localized at the genital ridge. These data may have use in practical aquaculture as well as in research to investigate germ-line chimerism in tench.

Introduction

The tench *Tinca tinca* (L.) is a teleost (Cyprinidae), closely related to common carp *Cyprinus carpio*, goldfish *Carrasius auratus*, and zebrafish *Danio rerio* (Nelson, 2006). It is traditionally reared in ponds of Central and Eastern Europe (Gela et al., 2007) and Asia (Wang et al., 2006) and is also being cultured in North America and Africa (Nelson, 2006). The demand for tench fry and stock for aquaculture has been increasing (Podhorec et al., 2011), and research has been conducted on all aspects of their artificially controlled reproduction.

Developmental stages of tench are described in a way similar to those published for zebrafish (Kimmel et al., 1995), goldfish (Yamaha et al., 1999), and loach *Misgurnus anguillicaudatus* (Fujimoto et al., 2004, 2006). Tench development has been reported by Penaz et al. (1981), as well as by Korzelecka-Orkisz et al. (2009) who also studied the effects of temperature on the duration of tench embryogenesis but did not focus on individual stages. Therefore our study was devoted to a more detailed characterization of each developmental stage to describe the germ cell lineage, a study not previously conducted in tench.

Primordial germ cells (PGCs) are embryonic cells that possess the potential to transmit genetic information to the next generation, and can be used for inter- and intra-specific transplantation. They have a potential for use in gene banking and cryopreservation, particularly through the production of donor gametes leading to germ-line chimeras (Okutsu et al., 2006; Yamaha et al., 2007). This technique can make possible the production of gametes of endangered species by using more common species as surrogate hosts (Okutsu et al., 2006; Yamaha et al., 2007). PGCs can be visualized by injection of synthetized mRNA with green fluorescent protein (GFP; Saito et al., 2006, 2011) for transplantation. The transplantation of blastomeres containing PGCs has been performed in fish species including zebrafish (Lin et al., 1992), medaka *Oryzias latipes* (Wakamatsu et al., 1993), rainbow trout *Oncorhynchus mykiss* (Takeuchi et al., 2001), goldfish (Yamaha et al., 2001), and loach *M. anguillicaudatus* (Nakagawa et al., 2002). A single PGC was first transplanted by Saito et al. (2008). To use this technique in tench, a detailed study of PGC development in tench embryos was needed.

The primary objective of this study was to describe the early developmental stages of tench to provide standardized staging for practical hatchery work and to enhance the scientific utility by linking the staging to germ-cell lineages. A secondary aim was to describe migration pathways of PGCs in tench as a tool for interpreting their development as a first step to applying germ cell transplantation technology to this species.

Materials and methods

Eggs and sperm

The eggs and sperm were collected from four female and two male tench, *Tinca tinc*a, body weights 535–1030 g and ages 9–15 years, reared in outdoor ponds. During the spawning season at the beginning of June 2012, fish were transferred to 4000-L indoor tanks with water temperature 20–22°C, at the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology (RIFCH) at the University of South Bohemia (SBU), Vodnany, Czech Republic for 2 days. During handling, the fish were anesthetized with clove oil (0.03 ml L^{-1} water; Dr. Kulich Pharma, s.r.o., Czech Republic). Spermiation was stimulated by a single intramuscular injection of carp pituitary powder dissolved in 0.9% NaCl solution at 1.5 mg kg^{-1}

Table 1
Cleavage and blastula of tench embryos at 20°C – development comparison of embryos from two females

Stage	Time		Characteristics	Fig. No.
	Female 1	Female 2		
Cleavage				
1-cell	60 min	50 min	Beginning of cleavage	1b
2-cell	1 h 15 min	1 h 10 min	1st cleavage furrow	1c
4-cell	1 h 50 min	1 h 40 min	2 × 2 array of blastomeres	1d
8-cell	2 h 35 min	2 h	2 × 4 array of blastomeres = 8 blastomeres on the yolk	1e
16-cell	2 h 55 min	2 h 30 min	4 × 4 array of blastomeres - form an irregular square	1f
32-cell	3 h 20 min	2 h 50 min	Irregular shape of blastodisc with 32 cells allocated to the central part of the animal pole	1g
64-cell	4 h	3 h 10 min	Blastodisc becomes a bi-layer of cells	1h
Blastula				
128-cell	4 h 20 min	3 h 40 min	Blastomeres arranged as a mound of cells adjacent to the yolk	1i
256-cell	4 h 50 min	4 h 10 min	Blastomeres increase in size	1j
512-cell	5 h 20 min	4 h 40 min	Hemispherical shape of blastodisc	1k
1k-cell	6 h	5 h	11 tiers of blastomeres	1l
1k-2k-cell	6 h 40 min	5 h 20 min		1m
Oblong	8 h 40 min	–	Blastodisc begins to flatten, ellipsoidal shape,	1n
Sphere	9 h 40 min	–	Spherical shape, border between blastodisc and yolk becomes flattened, beginning of epiboly	1o
Dome – 30% epiboly	10 h 40 min	–	Doming of the yolk cell, 30% of the yolk covered by the blastoderm	1p

Table 2
Gastrula, segmentation, and hatching of tench embryos at 20°C

Stage	Time	Characteristics	Fig. No.
Gastrula			
50% epiboly	12 h	50% coverage of yolk by blastoderm	1q
75% epiboly	14 h 40 min	75% coverage of yolk by blastoderm, elongated embryos according to animal-vegetal axis	
90% epiboly	16 h	Appearance of yolk plug	1r
100% epiboly	17 h 40 min	Tail bud prominent	1s
Segmentation			
2-somite	20 h	First somatic furrow	2a
4-somite	22 h 40 min		
6-somite	24 h 40 min	Brain primordium, optic vesicle	2b and 3a,b
8-somite	25 h 10 min		
11-somite	26 h 40 min	Optic vesicle enlarged	2c and 3c,d
16-somite	28 h 40 min		
18-somite	29 h 40 min		
20-somite	30 h 40 min	Formation of otic placodes, HTA = 135°	2d and 3e
24-somite	32 h 40 min	Hollowing of otic placodes to vesicles	3f
26-somite	33 h 40 min	Tail bud stage, HTA = 130°	2e
28-somite	34 h 40 min		
30-somite	35 h 40 min	Elongation of tail bud, HTA = 120°	2f
32-somite	37 h 40 min	Formation of optic cup and lens placode	
35-somite	38 h 40 min	HTA = 115°	2g and 3g,h
40-somite	40 h 40 min	Median fin fold, appearance of heart, HTA = 105°	2h
43-somite	48 h 40 min	Weak heartbeat	
45-somite	57 h 40 min	Blood circulation, stronger heartbeat, HTA = 75°	2i and 3i,j
Hatching			
Completion of somitogenesis	65 h	Hatch, appearance of pigmentation in retina and skin, HTA = 45°	4a
1st fry	98 h	Appearance of pigment over entire body, red-coloured heart	4b,c
2nd fry	110 h	Small pectoral fins	4d,e
3rd fry	152 h	Clear reduction in yolk size	4f,g
4th fry	188 h	Larger pectoral fins, appearance of swim bladder	4h,i
End of observation	212 h	Large swim bladder, swimming	4j,k

HTA, head-trunk angle.

body weight (BW). Sperm was collected 25 h post-injection into a 5 ml syringe containing 3 ml of immobilization medium Kurokura 180 (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, 340 mOsm kg^{-1}) (Rodina et al., 2004) and kept on ice (0–4°C) before use. The immobilization medium was used to facilitate short-term storage of sperm exposed to urine. Ovulation was induced by a single intramuscular injection of Ovopel [Prof. L. Horvath, University of Agricultural Sciences, Hungary, (Das, 2004)] dissolved in 0.9% NaCl solution at 54 mg kg^{-1} BW. Eggs were collected 29 h after the hormone injection. Eggs were inseminated with heterosperm of two males diluted in tap water at 20°C for 3 min. The eggs were dechorionated immediately after fertilization with Ringer's solution (112.2 mM NaCl, 3.35 mM KCl, 2.27 mM $CaCl_2.2H_2O$, 23.81 mM $NaHCO_3$) containing 0.1% trypsin and 0.4% urea for 5–10 min. Dechorionated eggs were transferred to 50 ml of an initial culture solution containing Ringer's solution with 0.01% penicillin and streptomycin (Sigma-Aldrich, Prague, Czech Republic) in glass petri dishes (95 mm diameter × 10 mm) coated with 1.0% agar and incubated at 20°C (Q-cell, Czech Republic). Temperature control range was 20 ± 2°C throughout the experiment. The dechorionated eggs of each of the four females were divided into three petri dishes,

Table 3
Diameter of tench embryos immediately post-fertilization

Diameter of embryo (μm) Female No.	Mean ± SD
1	$938.37^{b} \pm 15.12$
2	$886.93^{a} \pm 24.71$

Measurements shown as means ± standard deviations (SD) for two females. Values with different letters are significantly different (ANOVA, Tukey's HSD test, $P < 0.05$).

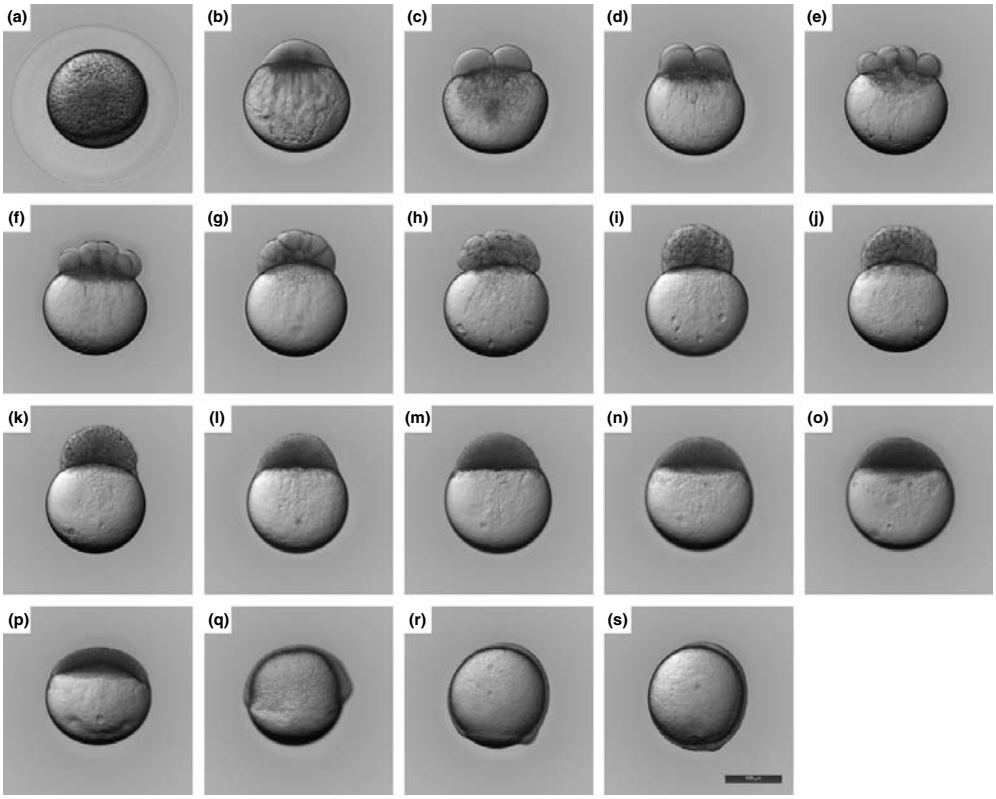

Fig. 1. Embryonic development of tench during cleavage, blastula, and gastrula at 20°C. (a) tench egg before dechorionation; (b) 1-cell stage, blastodisc formation; (c) 2-cell stage; (d) 4-cell stage; (e) 8-cell stage; (f) 16-cell stage; (g) 32-cell stage; (h) 64-cell stage; (i) 128-cell stage; (j) 256-cell stage; (k) 512-cell stage; (l) 1k-cell stage; (m) 1k-2k-cell stage; (n) oblong stage; (o) sphere stage; (p) dome stage, 30% epiboly; (q) 50% epiboly; (r) 90% epiboly; (s) 100% epiboly. Scale bar = 500 μm

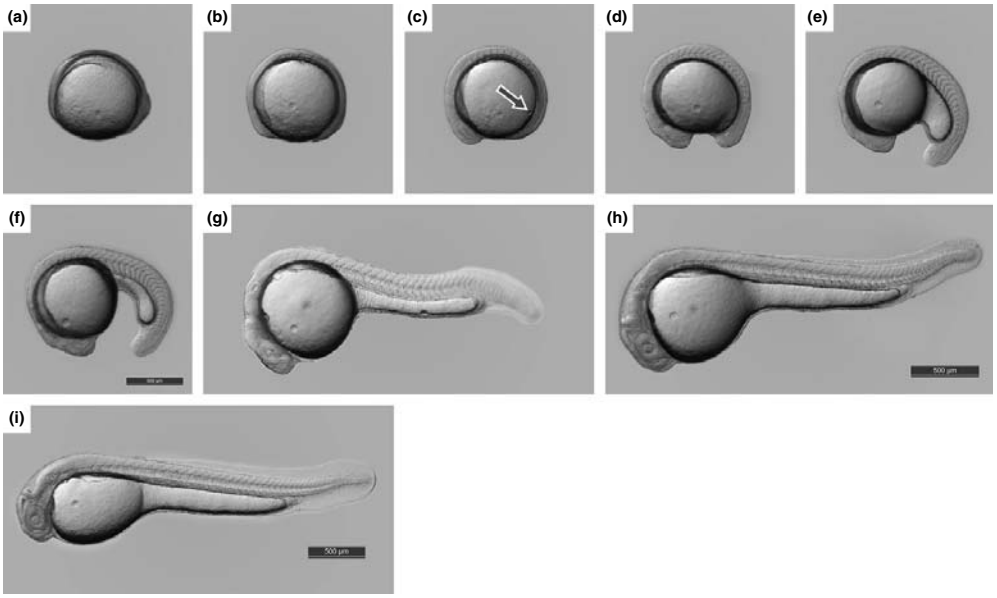

Fig. 2. Embryonic development of tench during segmentation at 20°C. (a) 2-somite stage; (b) 6-somite stage; (c) 11-somite stage, arrow = Kuppfer's vesicle; (d) 20 somite-stage; (e) 26-somite stage; (f) 30-somite stage; (g) 35-somite stage; (h) 40-somite stage; (i) 45-somite stage. Scale bars indicate 500 μm. Figures with same magnification: a–f; g and h; i

with each dish containing up to 20 individuals. After epiboly, embryos were transferred to a second culture solution under similar density parameters and volume, but with the addition of 1.8 mM $MgCl_2$ and 1.8 mM $CaCl_2$, treated with antibiotics, and incubated at 20°C. The culture solutions were changed daily.

Stage definition

Embryo developmental stages were designated according to morphological characteristics determined by external appearance under stereomicroscopy (Leica M165FC, Wetzlar, Germany) and from photographic records (Leica DFC425C). Developmental stages were documented at regular intervals: cleavage period, every 10 min; blastula, gastrula, and segmentation periods, every 30 min to 1 h; hatching period every 6 h. The segmentation period was described more accurately by recording the number of somites and the head-trunk angle (HTA) as a complementary angle to the angle created by a line drawn through the center of the ear and eye and a second line parallel to the notochord at the 5–10 somites level of the developing embryo, according to Kimmel et al. (1995).

Detection and observation of primordial germ cells

Primordial germ cells were visualized with synthetized mRNA containing GFP, and their migration routes documented beginning at 100% epiboly to the conclusion of hatching, using a fluorescence stereomicroscope (Leica M165FC) and photographic records (Leica DFC425C). The embryos were kept in darkness at 20°C in an incubator (Q-cell, Czech Republic) to avoid attenuation of fluorescence of GFP, as described earlier. A non-injected control group was kept under similar conditions.

Construction and synthesis of mRNA

RNA was generated from DNA plasmids (Saito et al., 2006), transformed into *Escherichia coli* (One Shot® TOP10; Life Technologies, Prague, Czech Republic), spread onto agar plates with ampicillin (Life Technologies), and incubated at 37°C for 16 h. Bacteria resistant to ampicillin (white intact colonies) were checked by direct PCR. DNA was isolated using the Plasmid Maxi Kit (Qiagen, Hilden, Germany). Amplified plasmid DNA was digested with Xba1 enzyme (Invitrogen) according to the manufacturer's instructions and purified by Phenol-Chloroform Isoamyl Alcohol (PCI, Sigma-Aldrich) DNA Extraction. Capped sense GFP-*nos*1 3′UTR mRNA was synthesized using the mMESSAGE mMACHINE kit (Ambion by Life Technologies) according to the manufacturer's protocol. For injection, the synthesized mRNA was diluted to 300 ng ul^{-1} in 0.2 M KCl.

Microinjection of mRNA

The mRNA was injected by micropipette into the yolk just under the blastodisc of tench embryos at the 1-4-cell

stage. The glass micropipette was drawn from a glass capillary tube (Drummond) using a needle puller (PG-10; Narishige, Tokyo, Japan). Microinjection was performed using a micromanipulator (Narishige) with micro-injector (Eppendorf, Hamburg, Germany) under a stereomicroscope (Leica M165FC).

Histology

The position of the PGCs was determined from histological sections of larvae after hatching. Eight days post-fertilization, larvae were fixed in Bouin's fixative (Sigma) for 3 h and stored in 80% ethanol at 4°C. Larvae were dehydrated in an acetone-xylol series, embedded in paraffin blocks, cut into 8 μm sections (Microm, Germany), and stained with haematoxylin-eosin according to standard procedures. PGCs were identified on the basis of their histological characteristics and location at the site of future gonads by optical microscopy (Olympus BX41, Tokyo, Japan).

Results

Embryonic development in tench

Developmental stages of tench were defined based on morphological features by comparison with the developmental stages of zebrafish and divided into five periods: cleavage, blastula, gastrula, segmentation, and hatching.

Eggs of two females (1 and 2) were monitored to compare timing of development during cleavage and beginning of the blastula. The development timing of the two egg groups was asynchronous (Table 1), with differences also seen among embryos within a group. Observations of embryos from female 1 continued from the late blastula period through the end of the hatching period (Table 2). We also measured the size of embryos of these two groups immediately after fertilization and compared it with the course of development (Table 3). The embryos of female 1 were larger and cleaved sooner than embryos of female 2. DNA analysis of embryos from each group confirmed that all were 2*n* (data not shown).

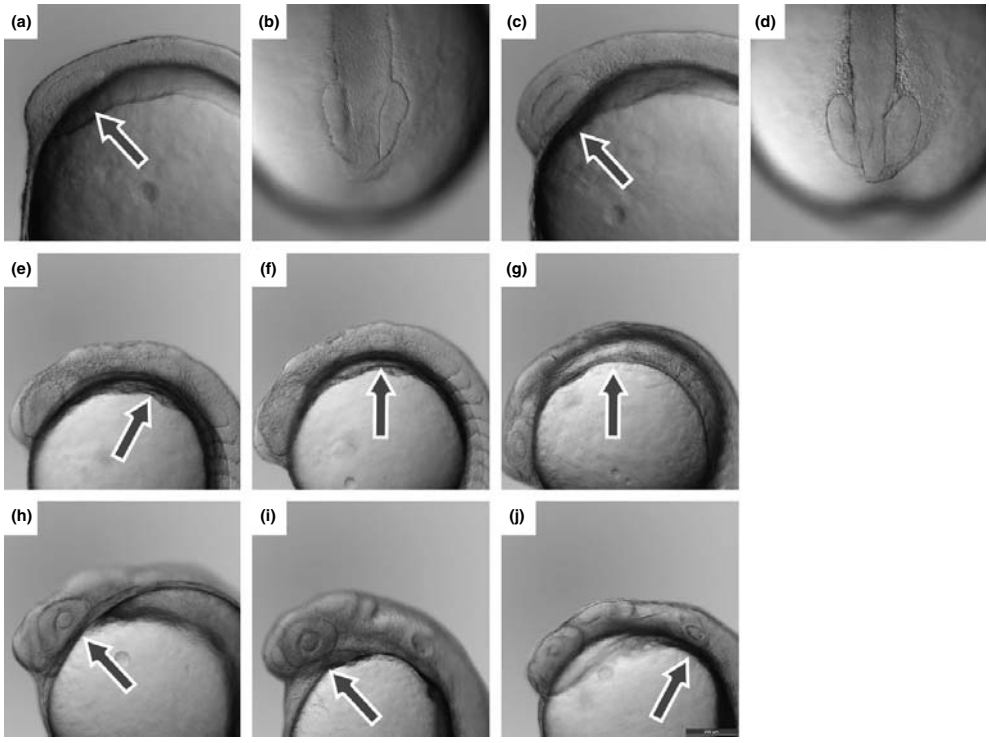

Fig. 3. Development of optical and auditory organs in tench at 20°C. (a, b) 6-somite stage, formation of the optic primordium; (c, d) 11-somite stage, enlargement of optic vesicles, formation of horizontal crease in center of optical organ; (e) 20-somite stage, formation of otic placode; (f) 24-somite stage, otic vesicle formation; (g) 35-somite stage, enlargement of the cavity of otic vesicle; (h) 32–35-somite stage, formation of the optic cup and lens; (i) 45-somite stage, appearance of pigmentation in the retina; (j) 42–45 somite stage, formation of otoliths in the otic vesicle. (a, c, e, f, g, h, i, and j) are lateral and (b, d) animal pole views. Scale bar = 200 μm. Arrows = optical or auditory organs

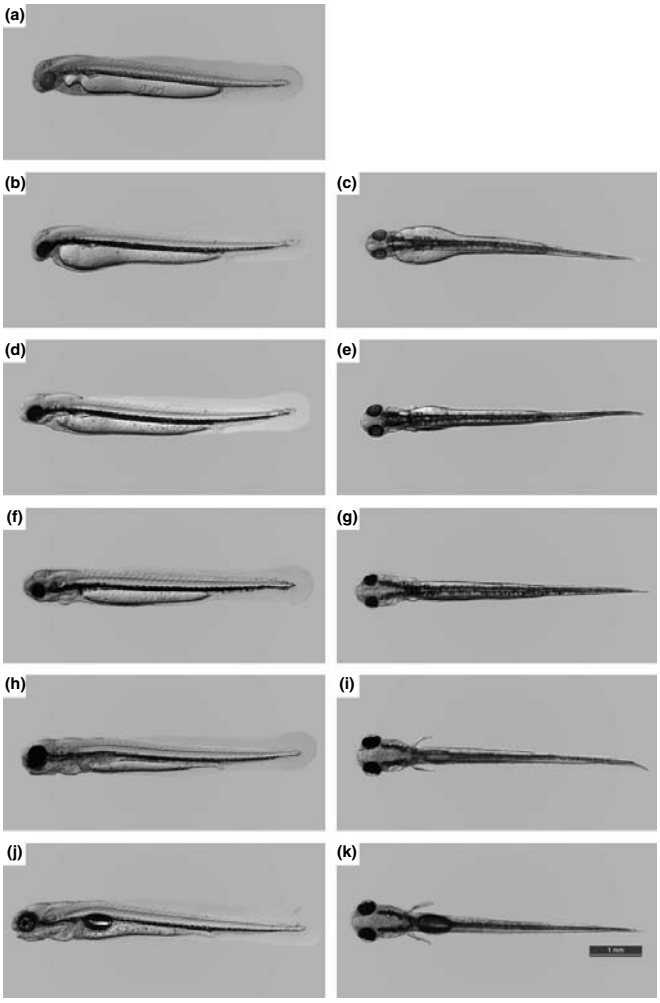

Fig. 4. Embryonic development of tench during hatching at 20°C. (a) 50-somite stage, hatching; (b, c) 1st fry stage; (d, e) 2nd fry stage; (f, g) 3rd fry stage; (h, i) 4th fry stage; (j, k) fry with large swim bladder, end of observation. (a, b, d, f, h, and j) are lateral and (c, e, g, i and k) are dorsal views. Scale bar = 1 mm

Cleavage period

After insemination and activation by contact with fresh water, the chorions of the eggs lifted away from the fertilized eggs to produce a perivitelline space that blocked polyspermy (Fig. 1a). The blastodisc was formed at 50–60 min post-fertilization (mpf; Fig. 1b). The cells divided synchronously at the animal pole during cleavage, but differed in timing at subsequent stages of this period (20–60 min between stages). The cleavage period was measured from the two to 64-cell stage (Table 1; Fig. 1b–h).

Blastula period

The blastula period was divided into early and late stages and started when the blastodisc became spherical. The mid-blastula transition (MBT) occurred during this period (Fujimoto et al., 2004) as a transition from synchronous to asynchronous cleavage and cell movement. The early blastula (morula) was characterized by synchronous cleavage at 128-, 256-, 512-, and 1k-cells. The late blastula, with asynchronous cleavage, consisted of oblong, sphere, and dome (30% epiboly) stages. Movement of the yolk syncytial layer toward the

Table 4
PGCs development of tench at 20°C

Stage name	Time	Characteristics	Fig. No.
Cleavage period			
1-cell	1 h	Beginning of cleavage	
Gastrula period			
100% epiboly	18 h	Appearance of PGCs	5a,b
Segmentation period	20 h		5c–f
6-somite	24 h		
13-somite	27 h	Migration of PGCs axial-ward	5g,h
20-somite	30 h		5i,j
28-somite	33 h	Appearance of PGCs under the trunk	5k,l
40-somite	40 h	Migration of PGCs to yolk extension	
43-somite	46 h		5m,n
45-somite	52 h		5o,p
Hatching period			
50-somite	58 h–64 h		6a,b
1st fry	100 h	Migration of PGCs to posterior parts	
2nd fry	112 h		6c,d
	136 h	Counting number of PGCs in 15 larvae	7a–h
3rd fry	160 h		6e,f
4th fry	184 h	Location of PGCs in genital ridge	6g,h
End of observation	208 h		

vegetal pole was identified at around the sphere stage (Fig. 1o) and signaled the beginning of epiboly. The embryos began to orient with the animal pole upward, compared to previous stages. From the dome stage (30% epiboly) we defined the progress in development as percent epiboly, indicated by the proportion of the yolk cell surface covered by the blastoderm (Table 1; Fig. 1i–p).

Gastrula period

During this period (Table 2; Fig. 1q–s) involution, convergence, and extension occurred. This began at 50% epiboly when the germ ring formed a ring at the blastoderm margin, the convergence movements produced a local accumulation of cells at the embryonic shield on the dorsal side, and hypoblast and epiblast lineages of the embryos were established. Gastrula concluded when the yolk cell surface was covered by the blastoderm (100% epiboly; Fig. 1s).

Segmentation period

This period comprises the development of somites (Table 2; Fig. 2), which appeared sequentially from the trunk to the tail, providing a tool to describe the stage by counting the number of somites appearing per hour. The somites consisted of mesodermal cells that elongated into muscle fibres on both sides of the neural tube. The tail bud became more prominent, the body of the embryo elongated and primary development of organs occurred. The development of optical and auditory organs was documented (Fig. 3) and used for identification of the stage. Morphological characterization of this period was completed by measuring the HTA of embryos from the 20-somite stage, including the first observation of auditory organs, to the completion of somitogenesis, according to Kimmel et al. (1995) (Table 2). The earliest-appearing somites developed slightly more rapidly than the later, and the number of somites increased on average by 2–4 pairs every 2 h, with the number of somites reaching 47–55 pairs. The first somatic furrow formed in the center of the dorsoventral part of the embryo at 19–20 hpf (Fig. 2a). The optic primordium and Kuppfer's vesicle appeared at the 6-somite stage (Fig. 3a,b). At the 11-somite stage, the optic vesicles enlarged and formed a horizontal crease in the center of the optical organ (Fig. 3c,d). A pair of small otic placodes formed adjacent to the hind-brain at the 20-somite stage (Fig. 3e) and developed into otic vesicles at the 24-somite stage at 33 hpf (Fig. 3f). The volume of the otic vesicles cavity increased (Fig. 3g). The optic vesicle began to develop into optic cups at the 32-somite stage, and lenses were clearly visible in the hollow in the center of the optic cup at the 35-somite stage (Fig. 3h). The retina began to pigment slightly (Fig. 3i). Two otoliths developed in each otic vesicle at the 42-45-somite stage (Fig. 3j).

Hatching period

Hatching was monitored from the 50-somite stage until the embryonic swim bladder appearance at 4th fry stage (Table 2; Fig. 4). Somitogenesis was complete at approximately 65 hpf, and embryos began hatching asynchronously among the experimental batches. For up to 1 h post-hatching, larvae remained at the bottom of the petri dish, before becoming motile and beginning to swim. At 212 hpf we observed a large swim bladder and the larvae swam and began active feeding (Fig. 4j,k).

Development of PGCs

Visualization of PGCs. Visualization of PGCs was achieved by injection of GFP-*nos*1 3′UTR mRNA into embryos of two females. In total, 183 embryos were injected and 69.3% showed a strong GFP signal for PGCs. Compared to controls, there were only small differences in development between injected (59.7% developed normally) and non-injected embryos (67.2%). The positions of PGCs were identified at regular intervals (Table 4). Because appearance of natural pigments in larvae made detection of PGCs more difficult during the hatching period, monitoring was discontinued at 9 dpf.

The first PGCs were observed at 18 hpf, just prior to the embryos reaching 100% epiboly (Fig. 5a,b). Before this period, PGCs were not detected, since the background expression of GFP in somatic cells was high, and the PGCs could not be distinguished from somatic cells. The number and position of fluorescence cells differed among embryos. At the appearance of the first somites, PGCs usually localized on both sides towards the caudal region of the dorsal, rarely on only one side, and began to migrate from the medial to the posterior position (Fig. 5c–f). During the 10–20 somite stage,

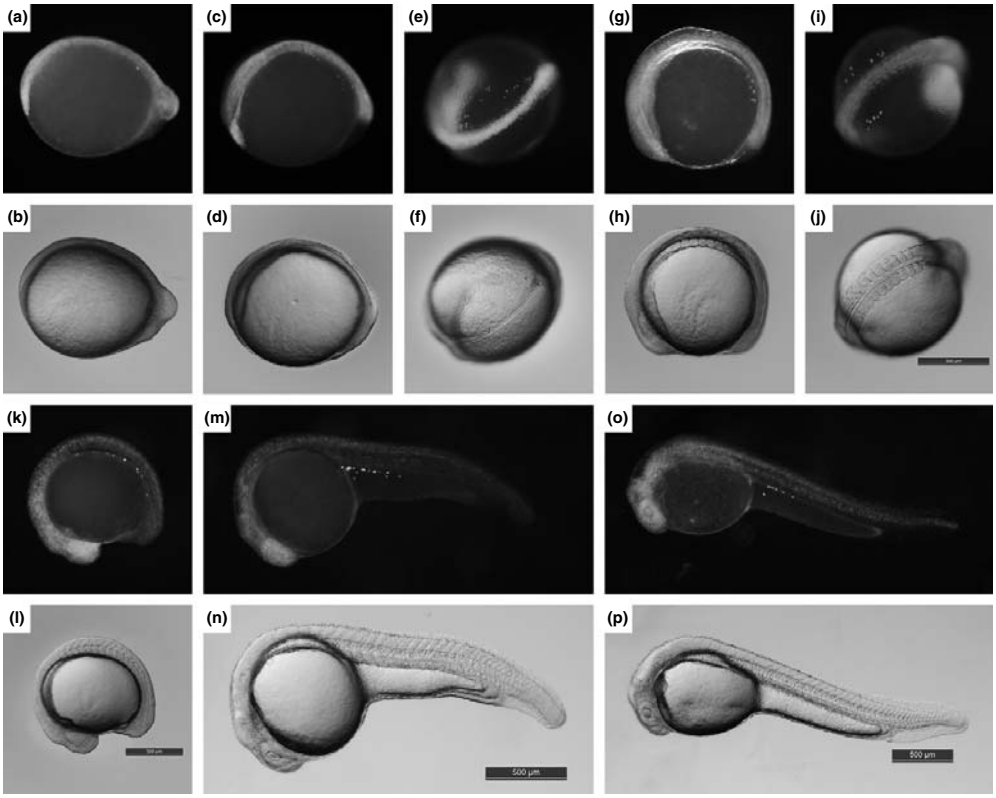

Fig. 5. Primordial germ cell development of tench during gastrula and segmentation at 20°C. (a, b) 100% epiboly; (c–f) beginning of segmentation; (g–j) 8-20-somite stage; (k, l) 28-somite stage; (m, n) 43-somite stage; (o, p) 45-somite stage. (a, c, e, g, i, k, m, and o) show fluorescence; (a–d, g, h, k, l, m–p) are lateral and (e, f, i and j) dorsal views. Scale bars = 500 μm. Figures with same magnification: a–j; k and l; m and n; o and p

PGCs loosely aligned on both sides of the somites from the 5th somite to the posterior of the developing embryo (Fig. 5g–j) and, near the 28-somite stage, began to appear in more posterior positions (around the 15th somite; Fig. 5k,l). At the 40–43 somite stage, after the tail separated from the yolk, the PGCs localized at the junction of the yolk ball and yolk extension as it developed (Fig. 5m,n) and remained at this position in the newly hatched larvae (Fig. 5o,p).

During the hatching period, the PGCs reached the central part of the yolk extension (Fig. 6a–d), localized to the ventrolateral of the alimentary canal, and moved dorsally. The PGCs did not change position rapidly and did not aggregate. Most of the PGCs localized in the lateral and posterior regions of the swim bladder, and the remainder to the posterior of the larva body at the 3rd (Fig. 6e,f) and 4th (Fig. 6g,h) fry stages. Finally, most of these cells migrated to the upper part of the gut at the genital ridge. The number of GFP-positive cells was counted and their positions were identified in 15 larvae at 6 dpf. At this stage, the number of GFP-positive cells in each larva was 3–10 (mean 8.1), with 95.1% localized around the genital ridge and 4.9% in an ectopic position (Fig. 7).

PGC identification by histology

The positions of PGCs were detected in the larvae at 8 dpf (Fig. 8). The PGCs were identified in histological sections by their characteristics and position. Most PGCs were large and spherical with a nucleus surrounded by a clear nuclear membrane. The PGCs were identified as histologically in the same positions as observed with fluorescent microscopy. Whole larvae were cut into serial sections, and PGCs were found in the body cavity between the swim bladder and gut dorsal to the intestine where the genital ridge is assumed to be localized. According to the positions of PGCs in the histological section, we confirmed that GFP-positive cells are PGCs.

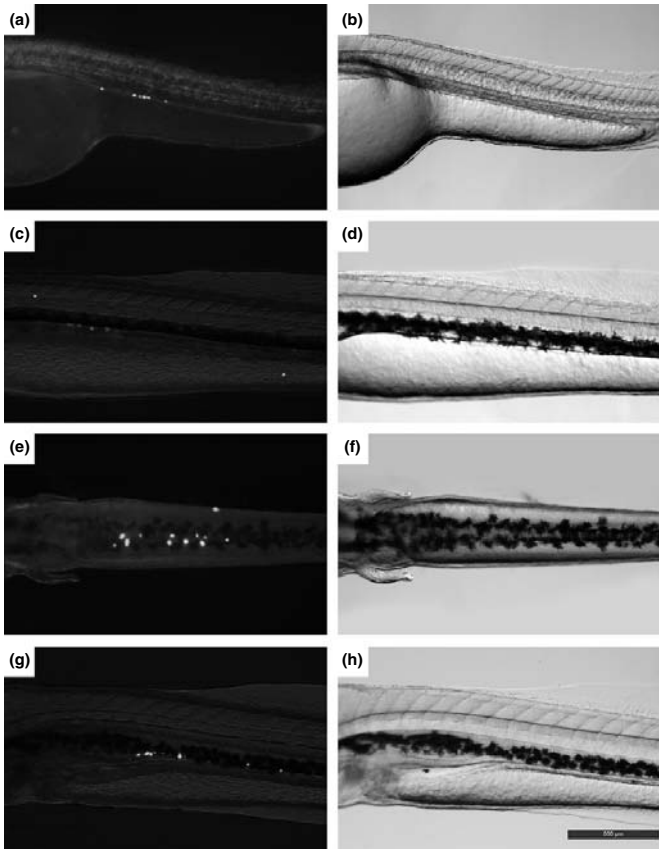

Fig. 6. Primordial germ cell development of tench during hatching at 20°C. (a, b) 50-somite stage; (c, d) 2nd fry; (e, f) 3rd fry; (g, h) 4th fry; (a, c, e and g) show fluorescence, (a–d, g and h) are lateral and (e, f) dorsal views. Scale bar = 500 μm

Discussion

Embryonic and larval development in tench

The present study demonstrated that tench development follows the general chain of events characteristic of teleosts (meroblastic cleavage) such as zebrafish (Kimmel et al., 1995), ice goby *Leucopsarion petersii* (Arakawa et al., 1999), goldfish (Yamaha et al., 1999), and loach (Fujimoto et al., 2006). Developmental stages were determined according to Kimmel et al. (1995).

The timing of embryogenesis is not consistent and is temperature-dependent, with longer duration at lower water temperatures and shorter at higher temperatures (Korzelecka-Orkisz et al., 2009). The present study examined the tench development only at the optimal temperature of 20°C. The first cleavage appeared 50–60 mpf in our study compared to 45 mpf reported by Korzelecka-Orkisz et al. (2009) at the same temperature, indicating that timing of embryogenesis presents inter-individual variations. We also observed variation in cleavage timing among embryos derived from a single egg batch. Eight blastomeres were visible at 120 and 155 mpf in the present study compared to 90 mpf found in tench by Korzelecka-Orkisz et al. (2009), 75 mpf in zebrafish cultured at 28.5°C (Kimmel et al., 1995), and 120 mpf in loach (20°C; Fujimoto et al., 2006), showing similarity in timing of early embryogenesis of tench (our study) and loach.

During the segmentation period, we used the number of somites to define developmental stages as in previous studies (Kimmel et al., 1995; Arakawa et al., 1999; Fujimoto et al., 2006), because of their clear appearance and ease of counting, and also used a head-trunk angle (Kimmel et al., 1995) to obtain more precise data. The final number (47–55) of somites was roughly similar to the 50 reported in loach (Fujimoto et al., 2006) and 55 in *Misgurnus fossilis* (Kostomarova, 1991), but dissimilar to the number in zebrafish (30–34; Kimmel et al., 1995) and ice goby (36; Arakawa et al., 1999). At this developmental stage, organs such as optic and otic vesicles began to differentiate. The optic primordium appeared at around the 6-somite stage as is seen in zebrafish, loach, ice goby, and *M. fossilis*, and a pair of otic vesicles

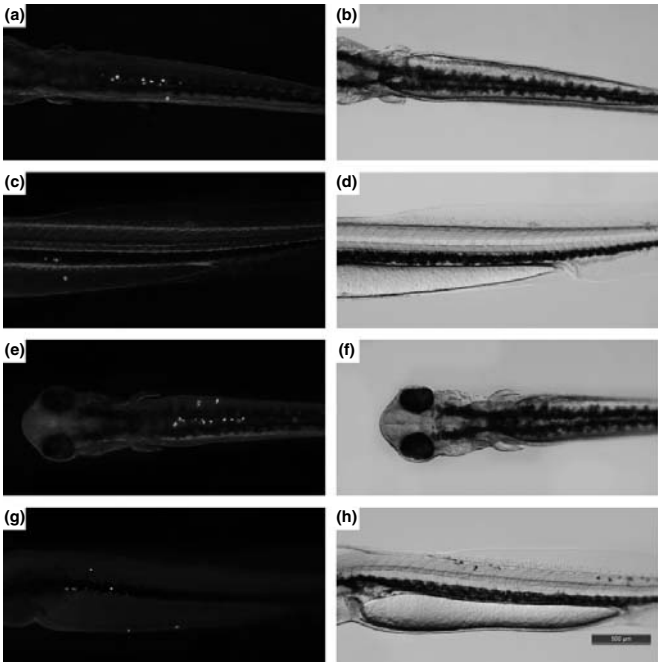

Fig. 7. Localization of primordial germ cells in two tench larvae during hatching at 20°C. (a–d) larva number 1; (e–h) larva number 2. (a, c, e, g) show fluorescence; (a, b, e, f) are dorsal and (c, d, g, h) lateral views. Scale bar = 500 μm

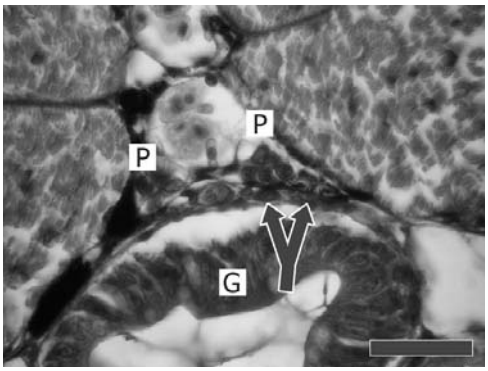

Fig. 8. Localization of primordial germ cells (PGCs) in transverse histological sections of tench larvae at 8 dpf. Haematoxylin-eosin stained transverse 8 μm sections, arrows = position of PGCs. Gut (G), pronephric duct (P). Scale bar = 100 μm

appeared at the 21-somite stage as in loach. Otic vessels are seen at the 10-, 20-, and 25-somite stages in zebrafish, ice goby, and *M. fossilis*, respectively.

Hatching of tench began shortly after somitogenesis as in zebrafish and goldfish, but loach are reported to hatch before the end of the formation of somites (Fujimoto et al., 2006). These comparisons indicate that embryonic and larval development is species-specific. A variation was found in the diameter of the tench embryos in the two analyzed groups before dechorionation (Table 3). Size differences among tench embryos are not unusual, and Korzelecka-Orkisz et al. (2009) documented that differences between the largest and smallest embryos reached nearly 70%.

PGCs development in tench

The origin of PGCs and their migration routes were examined by injecting synthesized mRNA containing a GFP sequence, a germ-line specific marker, into the fertilized eggs. The visualization of PGCs was lower (69.3%) than reported in ice goby (100%), medaka *O. latipes* (97.7%), goldfish (100%), zebrafish (100%), pearl danio *Danio albolineatus* (98.5%), loach (99.5%), and Pacific herring *Clupea pallasii* (100%; Saito et al., 2006). Compared to other fish species, the number of visualized PGCs was not high during the hatching period (average 8.1 labeled PGCs), possibly due to lower efficacy in labeling. This value might not reflect the true number of PGCs. In the other species, it has been shown that the number of visualized PGCs varied among embryos (Saito et al., 2006). Although the PGCs were counted at different stages of embryonic development, these data are comparable because PGCs do not proliferate during

their migration to the germinal ridge. Generally, GFP-positive cells do not appear prior to gastrulation (Saito et al., 2006, 2011). The germ cells were segregated from somatic lineages and migrated through tissues to reach the final positions in the genital ridge. The appearance of GFP-labeled tench PGCs was at 100% epiboly compared to other fish species (zebrafish, pearl danio, loach, Japanese eel *Anguilla japonica*), in which PGCs were observable at around 50% epiboly and in ice goby at 90% epiboly (Saito et al., 2006, 2011) mainly due to high background expression of GFP in all somatic cells. PGC formation was comparable to that observed in medaka, goldfish, and ice goby during the segmentation period (Saito et al., 2006). These results provide evidence that routes of PGC migration are species-specific. The position of PGCs (dorsolateral to the gut and ventral to the pronephric duct) found by histology was reported in histological sections of zebrafish larvae at 10 dpf (Yoon et al., 1997), confirming our fluorescently visualized cells as PGCs.

Our results indicate that the 3′UTR of the zebrafish *nos*1 mRNA can promote maintenance of RNAs in the PGCs. The visualization of PGCs tagged with GFP will be a powerful technique not only for further study of PGC formation, but also to investigate the potential of germ-line chimerism in tench.

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CHAPTER 4

ISOLATION AND CRYOPRESERVATION OF EARLY STAGES OF GERM CELLS OF TENCH (*TINCA TINCA*)

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Isolation and cryopreservation of early stages of germ cells of tench (*Tinca tinca*)

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ABSTRACT: A practical technique for isolation and cryopreservation of tench (*Tinca tinca*) (Cyprinidae, Teleostei) early stages of germ cells (GC), including spermatogonia and spermatocytes, is reported for the first time. The germ-line cells possess the ability to differentiate into functional gametes of both sexes. These early stages of germ cells are small enough to be well-suited to cryopreservation, which, together with their high level of plasticity, makes their preservation a promising tool for maintaining genetic resources. Testicular cells were distinguished and separated by Percoll gradient, with the highest proportion of GC (62.2%) obtained from the 30% layer. The concentration and viability of GC were determined, and specific staining (DDX4) for germ cells was used to distinguish GC from somatic cells. Early stages of germ cells were cryopreserved in an extender composed of phosphate buffered saline (pH 8) with 0.5% BSA, 50mM D-glucose, and containing 1.5M cryoprotectant in the pre-programmed PLANER Kryo10 series III using a cooling protocol from +10°C to –80°C at a rate of 1°C/min. The effect of six cryoprotectants – methanol, dimethyl sulfoxide, dimethyl sulfoxide + propanediol (1 : 1), glycerol, ethylene glycol, and dimethylacetamid was assessed, and the results were evaluated by comparing the percentage of viable frozen/thawed GC by ANOVA, Tukey's HSD test ($P < 0.05$). Almost the same viability rates were obtained with no significant differences among tested cryoprotectants, indicating high stability of GC in cryoprotectants. Nevertheless, glycerol at a concentration of 1.5M was associated with the highest survival rate of thawed tench GC (57.69 ± 16.85%).

Keywords: germ cells; isolation; transplantation; Percoll gradient; cryoprotectant; viability

INTRODUCTION

The germ-line cells, primordial germ cells (PGCs), and their subsequent stages, spermatogonia/oogonia (SG/OG), have the potential to undergo proliferation (Brinster 2002), differentiate into functional gametes, and transmit genetic information to the next generation (Okutsu et al. 2006b) making them suitable for xenogeneic transplantation (Yamaha et al. 2007). The major difference between manipulation of PGCs and SG is the relatively simple method of isolation of a large number of SG from testes, compared to the successful isolation of only tens of PGCs (Saito et al. 2008, 2010). A disadvantage of these early stages of germ cells (SG/OG and their subsequent stages) lies in limited migratory activity to the required position in the gonads when these cells are transplanted during embryonic stage. Therefore SG/OG must be transplanted into the peritoneal cavity near the genital ridge of hatched fry or directly into the gonads (Yoshikazi et al. 2010; Wong et al. 2011; Nobrega et al. 2012). The transplantation of PGCs/SG/OG into a closely related species,

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creating a germ-line chimera, could enhance fish production for research as well as aquaculture.

In recent years, the demand for tench *Tinca tinca* fry and stock for aquaculture has increased (Podhorec et al. 2011), and the preservation of tench sperm (Rodina et al. 2007) or germ cells may benefit artificial reproduction. Therefore tench, an economically important fish traditionally reared in ponds of Central and East Europe (Gela et al. 2007), was chosen for this project. Tench are closely related to common carp *Cyprinus carpio*, goldfish *Carrasius auratus*, zebrafish *Danio rerio*, and the white cloud mountain minnow *Tanichthys albonubes* (Nelson 2006), species that could possibly be used as hosts for xenogeneic transplantation.

The germline stem cells, including PGCs and SG, can undergo continued spermatogenesis after transplantation (Okutsu et al. 2006a). To successfully utilize the early stages of germ cells (GC) (including SG and several spermatocytes) for cryopreservation and later transplantation, they must be separated from other testicular cell types of mature testis as spermatids and mostly the sperm. Therefore a purification step is necessary. Several methods of GC isolation have been developed, including cell sorting via flow cytometry (Kobayashi et al. 2004), magnet-activated cell sorting (Schonfeldt et al. 1999), and density gradient centrifugation-elutriation (Loir and Le Gac 1994). The step Percoll gradient is a simple and economical method that has been used in several fish species (Lacerda et al. 2006; Psenicka et al. 2012) but has not been tested on tench.

Cryopreservation and cryobanking of fish gametes are considered tools for artificial reproduction and genetic improvement of aquaculture species. Cryopreservation provides several benefits, such as synchronizing the availability of male and female gametes, long-term storage of cells without aging, convenient transport of genetic material, and reducing the number of broodstock required in hatchery facilities and living gene banks (Suquet et al. 2000; Alavi et al. 2012). The process in fish sperm is relatively simple (Bobe and Labbe 2010) and has been used in more than 200 fish species (Billard and Zhang 2001). The disadvantage of sperm cryoconservation is that it does not provide for storing the maternal information. Oocytes and embryos have been successfully cryopreserved only in invertebrates (Labbe et al. 2013). The large size and high yolk content of fish oocytes and embryos results in slow penetration of the extender and high sensitivity to freezing (Zhang et al. 2007; Robles et al. 2009). The most effective alternative to cryopreservation of fish oocytes and embryos is the use of early stages of germ cells with their ability to re-colonize genital ridges upon transplantation (Yoshizaki et al. 2005, 2011; Okutsu et al. 2006b). Therefore GC are well-suited to cryopreservation, due to small size and a high level of sexual plasticity which allows them to differentiate into fully functional gonads of both sexes (Okutsu et al. 2006b, 2007).

The primary aim of this study was to investigate the optimal procedure for tench GC isolation and cryopreservation, focusing on cryoprotectant solution. This is the first report of tench early stages of germ cells isolation and preservation.

MATERIAL AND METHODS

The study was conducted in the aquaculture facility of the Faculty of Fisheries and Protection of Waters at Vodňany (University of South Bohemia in České Budějovice, Czech Republic).

Fish and rearing conditions. Tench, 2–2.5 years of age, were obtained from outdoor ponds at the Research Institute of Fish Culture and Hydrobiology at the beginning of July 2013, transported to the hatchery, and separated by sex according to sexual dimorphism. Twenty males (30.67 ± 6.71 g average body weight and 130.94 ± 10.41 mm average body length) were selected and transferred into two 50 l aquariums. Temperature and oxygen values were monitored daily and ranged 18–22°C and 6–7 mg/l, respectively. Fish were fed daily with pelleted feed; feeding was stopped 48 h before initiation of the experiment. Four males, producing spermatozoa, were used for each trial. Testicular cells from a single immature male (did not produce any spermatozoa) were examined using an immulocytological and immunoblotting method.

Adjustment of osmolality. The osmolality of tench blood plasma was determined to optimize conditions for germ cells. Blood samples (300 µl) were collected from the caudal vein of 4 fish using a 1 ml syringe containing 10 µl of heparin (Zentiva, Prague, Czech Republic) and held at 4°C until use. Osmolality of blood samples was assessed using a Vapour Pressure Osmometer Vapro 5520 (Wescor, Logan, USA), expressed in mOsm/kg, and used for adjusting the media for isolation and separation of testicular cells.

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Isolation and enzymatic dissociation of testicular cells. Four male tench (43.7–36.7 g body weight) were selected, killed by a blow to the skull, and testes (0.18 ± 0.02 g average weight) were dissected and transferred to individual Petri dishes. The lipid tissue was separated from the testes to avoid high amounts in the isolated cell suspension, and the testes were washed in phosphate buffered saline (PBS, 248 mOsm/kg, pH 8) (Sigma-Aldrich, s.r.o., Prague, Czech Republic), cut into small pieces, and transferred into 15 ml tubes with 10 ml of PBS + 0.1% each of collagenase (Life Technologies Czech Republic s.r.o., Prague, Czech Republic) and trypsin (Sigma-Aldrich) (248 mOsm/kg, pH 8) according to Psenicka et al. (2012). Testicular suspensions were incubated in a Compact Bio Shaker VBR-36 (Bionexus Inc., Oakland, USA) with gentle mixing for 1.5 h at 25°C. The enzyme activity was stopped, and DNA released from dead cells was digested by adding 1 ml of PBS containing 1% BSA (Sigma-Aldrich) and 40 µg/ml deoxyribonuclease (DNase) (AppliChem GmbH, Darmstadt, Germany), respectively. The homogeneous suspension was filtered through a 50 µm filter (Partec GmbH, Görlitz, Germany). To separate GC from other testicular (spermatids and sperm) and somatic cells, a step Percoll gradient (Sigma-Aldrich) 30 and 5% (in the order from the bottom of tube) was prepared. The testicular cell suspension was slowly transferred to the surface of the gradient and immediately centrifuged at 500 *g*, 4°C for 30 min with a slow rotor acceleration to preserve the Percoll gradient. 3 ml of cell suspension was collected from the 30% and 5% layers and the pellet (precipitate collected at the bottom of the tube), washed with 10 ml PBS (248 mOsm/kg, pH 8) and centrifuged at 500 *g*, 4°C for 30 min. Pellets with testicular cells were transferred into 1.5 ml Eppendorf tubes, diluted with PBS to 0.5 ml, and kept on ice at 4°C for analysis. The testicular cells from each layer of Percoll gradient and pellet were confirmed by light optical microscope Olympus BH2 (Olympus Corp., Tokyo, Japan) at 250× magnification and photographed by Nikon 5100 camera (Nikon, Tokyo, Japan) (Figure 1). The GC were discriminated from small testicular cells (spermatids and sperm) and components (lipid, debris) on the basis of their spherical appearance, large size, and large nucleus with small nucleolus. The number of cells was evaluated using Olympus MicroImage software (Version 4.0 for Windows 95/NT/98).

Evaluation of GC concentration. GC concentration in whole testes of each fish was determined microscopically by counting the number of cells diluted in PBS (dilution 1 : 10) using a Burker cell

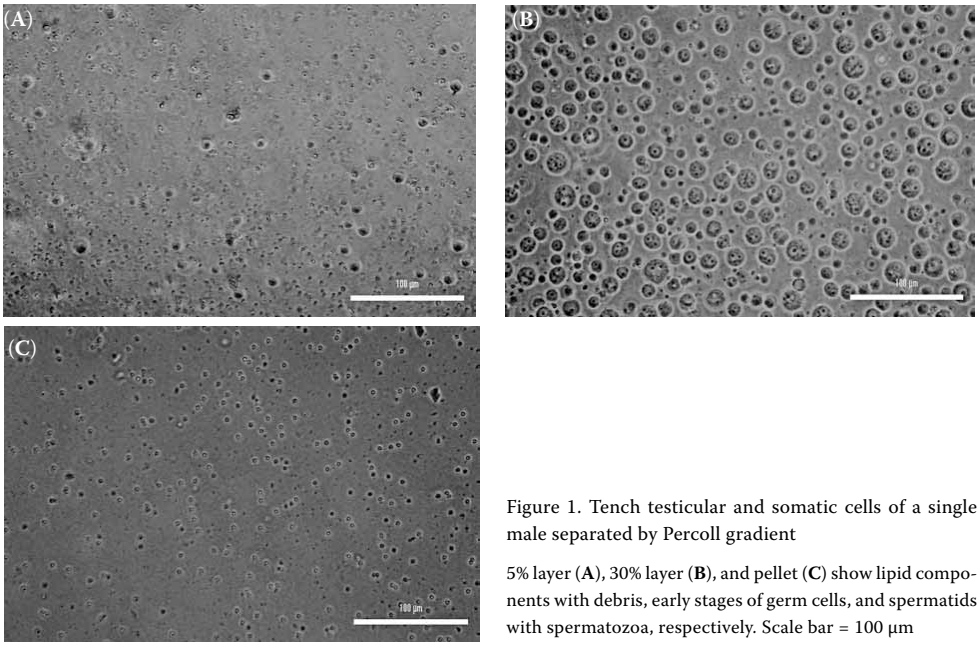

Figure 1. Tench testicular and somatic cells of a single male separated by Percoll gradient

5% layer (**A**), 30% layer (**B**), and pellet (**C**) show lipid components with debris, early stages of germ cells, and spermatids with spermatozoa, respectively. Scale bar = 100 µm

hemocytometer (Meopta-optika, s.r.o.,, Přerov, Czech Republic) at 100× magnification under an Olympus BH2 microscope. The number of GC was counted in 20 squares of the Burker cell chamber with two repetitions, and the GC content was expressed as the absolute number of GC obtained from testes of each male by multiplying the number of GC by the volume of each GC sample (0.5 ml).

Immunofluorescence labelling. A standard protocol for indirect immunofluorescence labelling was used to discriminate germ cells from somatic cells. Specific staining of germ-line cells with primary rabbit polyclonal antibody to DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; Cat. No. GTX116575; Lot No. 40261) (GeneTex Inc., Irvine, USA) at a dilution 1 : 300 combined with secondary antibody goat anti-rabbit immunoglobulin (IgG) conjugated with fluorescein isothiocyanate (FITC) (dilution 1 : 700) (Sigma-Aldrich, s.r.o., Prague, Czech Republic) was applied. DDX4 (known as VASA) was chosen as specific marker because it is the most widely used marker of the germ cell lineage and it has been identified in the germ cells of a large number of animals (Raz 2000; Bellaiche et al. 2014). To visualize all cells, the nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). As a control, somatic cells from caudal fins were examined. Slides with whole testis samples from each individual and each layer of the Percoll gradient and pellet were observed and recorded under an inverted fluorescent microscope Olympus IX 83 (Olympus Corp., Tokyo, Japan) with Hamatsu Digital camera C10600 (Hamamatsu Photonics, Hamamatsu, Japan) at 400× magnification. Percentage of GC obtained from the 30% Percoll gradient from a single immature male in which final stages of spermatogenesis did not start (no spermatids and sperm presented) was evaluated by cellSens Dimension Microscopy Imaging Software (Version 1.9, 2009–2013) and compared with data from light optical microscopy.

Immunoblotting. The Western blot was used to detect specific proteins in testis of immature tench male to confirm the specificity of DDX4 as a marker of germ cells in tench. Proteins were extracted from testis and somatic cells from fin (control) with lysis buffer (8M urea, 2M Thiourea, 4% CHAPS, 10% w/v isopropanol, 0.1% w/v Triton X-100, 100mM dithiothreithol; Sigma-Aldrich) containing phosphatase (1mM sodium orthovanadate, 50mM EDTA; Sigma-Aldrich) and protease (100mM PMSF, 1 µl/ml pepstain A, 5 µl/ml leupeptin; Sigma-Aldrich) inhibitors. The Bradford protein assay was applied to determine the protein concentration in samples. For SDS-PAGE the samples (25 mg of proteins) were resuspended in buffer containing 65mM TRIS, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, and 5% (vol/vol) beta-mercaptoethanol (Sigma-Aldrich), and denatured at 95°C for 3 min before loading onto gels. Proteins were separated on 12% gel. After electrophoresis the SDS gels were placed on polyvinyl difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, USA) and electrically transferred. The membranes were blocked by incubation with 5% (w/v) Skim milk in TBS-T (0.1% Tween-20, 20mM Tris, 500mM NaCl at pH 7.6) (Sigma-Aldrich) at 20°C for 1 h. The membranes were washed three times with TBS-T followed by incubation for 12 h at 4°C in 5% BSA-TBST containing anti-DDX4 antibodies as the primary antibodies. Then the membranes were washed and incubated with HRP-conjugated goat anti-rabbit IgG (1 : 3000 in 3% BSA-TBST) at 20°C for 1 h. Reacted proteins were revealed with 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich).

Cryopreservation and thawing procedures. GC samples from 4 tench male were treated individually. Before freezing, GC were diluted 1 : 3 (25 µl of GC diluted in PBS + 75 µl of extender) in an extender composed of PBS (pH 8) with 0.5% BSA, 50mM D-glucose (Psenicka et al. 2012), and 1.5M cryoprotectant to a final concentration of 8×10^5 of GC/ml. Six cryoprotectants were studied: (1) methanol (MET); (2) dimethyl sulfoxide (DMSO); (3) DMSO + propanediol (DMSO + P at 1 : 1); (4) glycerol (GLY); (5) ethylene glycol (EG); (6) dimethylacetamid (DMA). Tests were conducted in triplicate for each sample. Volumes of 100 µl diluted GC were placed into 1.8 ml cryotubes (Nunc, Roskilde, Denmark), transferred to a pre-programmed PLANER Kryo10 series III, and cryopreserved by cooling from +10°C to –80°C at a rate of 1°C/min. Time between GC sample treatment and initiation of freezing was 10 min. Frozen samples were transferred into liquid nitrogen (LN2) and stored for 7–14 days. To assess GC variables, samples were thawed by plunging the cryotubes into a 38°C bath until thawed up to 40 s. Finally, samples were centrifuged (500 *g*, 10 min, 4°C) for viability testing.

Evaluation of germ cell viability. The viability of GC from individual fish was tested prior to

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(in duplicate) and after cryopreservation/thawing (in triplicate). Twenty µl of GC were transferred into 1.5 ml Eppendorf tubes on ice at 4°C. Dual DNA staining to identify live and dead cells was performed using the Cellstain double staining kit (Sigma-Aldrich) according to the manufacturer's protocol. The staining was achievied by acetoxymethyl ester of calcein (Calcein-AM) (viable cells emit strong green fluorescence), and propidium iodide (PI), a nuclei staining dye which cannot pass through a viable cell membrane and stains DNA in degenerate cells (dead cells emit red fluorescence). The staining was optimized by adding PI at 5 µl/ml because of weak red fluorescent signal of tench GC. Samples were gently mixed, and 20 µl was pipetted onto a clean microscopic slide and left for 3–5 min for sedimentation. Ten images per sample were obtained using an Olympus BX 50 microscope (Olympus Corp.) at magnification 100×, recorded with a 3CCD Sony DXC-9100P colour camera (SONY Corp., Tokyo, Japan), and processed by Olympus MicroImage software (Version 4.0 for Windows 95/NT/98) according to Flajshans et al. (2004).

Data analysis. In cryopreservation and viability trials, cryoprotectants were tested in triplicate for each male, while GC concentration and osmolality were estimated from dual measurements. Data were calculated as means ± standard deviations (SD). To compare the percentage of viable GC with different cryoprotectant treatments, ANOVA with *post hoc* comparisons using Tukey's honest significant difference (HSD) test was applied. Probability values of $P < 0.05$ were considered significant. All statistical analysis was performed using STATISTICA software (Version 12, 2013) for MS Windows.

RESULTS

Adjustment of osmolality. Osmolality of blood samples (mean ± SD; $n = 4$) was 248 ± 6 mOsm/kg. The osmolality of the media used for experiments was adjusted to this level.

Isolation of early stages of germ cells in a Percoll gradient. The GC were separated from other testicular (spermatids and sperm) and somatic cells by Percoll gradient and identified on the basis of their morphological characteristics by light microscopy (Figures 1A–C) and by immunocytochemistry with specific staining for germ cells (Figure 2). GC were identified by immunolabelling, and the mean proportion in the 5% and 30% layer and pellet of the Percoll gradient from the four fish was calculated. Because the GC (spermatogonia and spermatocytes) are not easy to distinguish from each other by light microscopy, they were calculated together. Spermatids were counted together with sperm because of visible smaller sizes than GC. The upper layer in the Percoll gradient (5%) showed 80% lipids + debris and 20% GC. The 30% layer comprised 62.2% GC, 18% debris, 17.3% spermatids + spermatozoa, and 2.5% blood cells. The pellet consisted of 39.1% blood cells, 21.2% spermatids + spermatozoa, 20.7% debris, and 19% GC. Percentages of GC calculated from total amount of GC of 4 males in whole Percoll gradients were 25.9% in 5% layer, 50.1% in 30% layer, and 24% in pellet. Thus the highest concentration of GC was found in the 30% layer of Percoll gradient.

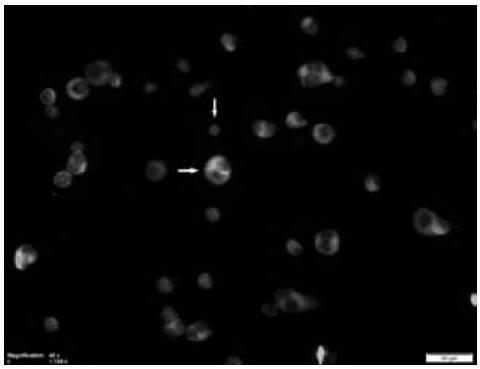

Figure 2. Fluorescent micrographs of tench testicular cells stained with DDX4 and DAPI

Specific staining with germ cell (GC) specific marker DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) indicates a strong green signal in early stages of GC and DAPI (4', 6-diamidino-2-phenylindole) blue in early stages of GC and somatic cells. Arrows indicate labelled GC (blue and green staining, left arrow) and somatic cell (blue staining, top arrow). Scale bar = 20 µm

GC concentration. The GC concentration was defined as the number of GC measured per unit (0.5 ml PBS) from each fish to determine the yield of GC from individual testes and specify the amount used for cryopreservation. The mean number of GC isolated from 0.18 ± 0.02 g testes of 4 males in two counts ranged from 1.31 ± 0.07 to 2.06 ± 0.13 × 10^6 (Table 1) with no significant differences ($P > 0.05$) among analyzed fish. The yield of GC of 2–2.5 year old tench was consistent among individuals.

Table 1. Yield of germ cells (GC) from testes (0.18 ± 0.02 g) of individual tench obtained from a 30% layer of Percoll gradient

Tench No.	Absolute numbers of GC (× 10^6 cells) (mean ± SD)
1	1.63[a] ± 0.19
2	2.06[a] ± 0.13
3	1.67[a] ± 0.17
4	1.31[a] ± 0.07

data are shown as means ± standard deviations (SD)
[a]not significantly different values (ANOVA, Tukey's HSD test, $P < 0.05$)

Immunocytochemistry and immunoblotting. Immunocytochemistry confirmed the identity of GC and distinguished them from somatic cells by staining with specific primary antibody to DEAD box protein. Germ cells were distinguished by a strong green fluorescent signal (Figure 2), while only a blue signal (DAPI) was observed in control somatic cells. The proportion of GC from the immature male isolated in the 30% Percoll gradient reached 92.9% (Figure 2) and confirmed that the 30% Percoll gradient is suitable for tench GC isolation, as shown with mature males. The Western blot technique confirmed the specificity of DDX4 as a suitable marker of tench germ cells. A bright band at 75 kDa was found at immunoblot (Figure 3) and proved that DDX4 is suitable for germ cell staining in tench.

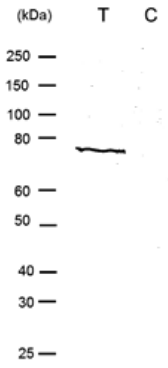

Figure 3. Immunoblot with proteins extracted from testis and caudal fin of tench male detected by DDX4

Bright band at 75 kDa indicates that DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) is a suitable marker of tench germ cells (T) in comparison with somatic cells (control, C) where no specific staining was documented

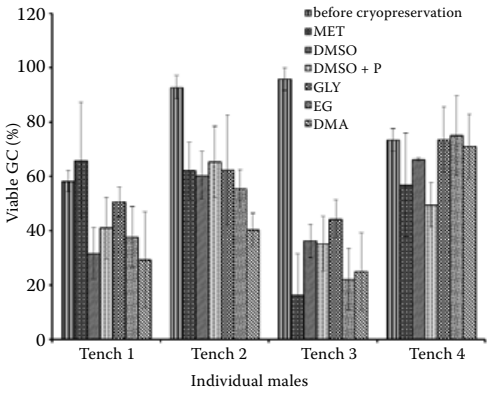

Figure 4. Percentage of viable early stages of germ cells (GC) before and after cryopreservation treated with six cryoprotectants in four fish

MET = methanol, DMSO = dimethyl sulfoxide, DMSO + P = DMSO + propanediol at 1 : 1, GLY = glycerol, EG = ethylene glycol, DMA = dimethylacetamid

Data are shown as means ± standard deviations for four individuals; viability was evaluated for six cryoprotectant treatments in duplicate before and in triplicate after cryopreservation

Viability of GC before and after cryopreservation with the effect of cryoprotectants. The efficacy of cryopreservation was evaluated by comparing the percentage of viable GC before and after cryopreservation. Decreases ($P < 0.05$) in percentage of viable GC were observed in all examined samples after cryopreservation (Figure 4). The mean numbers of viable GC from 2 analyses in four males were: 58.17 ± 3.93, 92.76 ± 4.34, 95.80 ± 4.20, and 73.45 ± 4.11% before cryopreservation (Figure 4) and (mean values of 6 cryoprotectant treatments and 3 replicates) 42.70 ± 18.58, 57.69 ± 14.50, 29.85 ± 14.71, and 65.36 ± 15.54% after cryopreservation.

The effects of six cryoprotectants were tested in four males. The percentage of viable GC varied among analyzed males and those cryopreserved with DMSO, EG, and DMA showed significant differences (Figure 4). The viability of GC from individual fish was consistent across the 6 cryoprotectants, suggesting that GC are individual-dependent (Figure 4). To detect the best cryoprotectant for cryopreservation of tench GC, the analyzed data from four tench males were averaged (Figure 5). The percentage of viable GC after thawing for the six cryoprotectants was: GLY 57.69 ± 16.85%, MET

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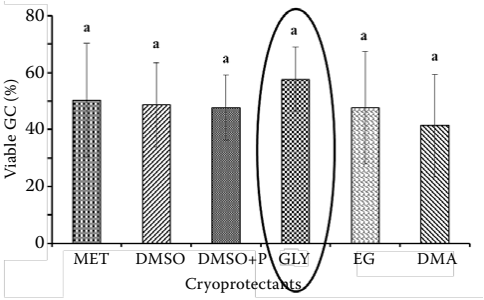

Figure 5. Percentage of viable early stages of germ cells (GC) after thawing with six cryoprotectants

MET = methanol, DMSO = dimethyl sulfoxide, DMSO + P = DMSO + propanediol at 1 : 1, GLY = glycerol, EG = ethylene glycol, DMA = dimethylacetamid

Effect of the cryoprotectants is shown as percentage of live GC after thawing of four fish with three replicates; the most effective cryoprotectant is circled. Results are shown as means ± standard deviations

[a]not significantly different values (ANOVA, Tukey's HSD test, $P < 0.05$)

50.27 ± 26.27%, DMSO 48.62 ± 16.49%, DMSO + P 47.77 ± 15.65%, EG 47.64 ± 22.76%, and DMA 41.38 ± 22.30%. ANOVA and Tukey's HSD test showed no significant differences among tested cryoprotectants. Nevertheless, glycerol was evaluated as the best cryoprotectant all over the four tested tench males for cryopreservation of GC, as it was associated with the highest viability of GC after cryopreservation (Figure 5).

DISCUSSION

Based on the results of the present study, the following can be suggested: (1) the Percoll gradient is a simple, inexpensive, and practical method of selecting testicular cells with the highest percentage of GC in the 30% layer; (2) staining with DDX4 indicates the presence of GC; and (3) addition of 1.5M glycerol to extender composition is the most suitable for freezing of tench GC.

Importance of germ cells. Germ cell transplantation to induce chimerism in fish species is an expanding focus of research. This technique can enhance the production of gametes of species that are commercially valuable, endangered, or have complex reproduction, using a more readily available species or one easily adapted to artificial reproduction as a surrogate host (Okutsu et al. 2006a; Yamaha et al. 2007). Primordial germ cell transplantation was developed (Saito et al. 2008, 2010) due to the advantage of their migration to genital ridge via chemotaxis. This characteristic was assumed to be lacking in early stages of germ cells as SG/OG (Raz and Reichman-Fried 2006), but this has been refuted by Okutsu et al. (2006a, b) and Yoshikazi et al. (2010), who argue that SG exhibit at least a low level of migration activity in response to chemotactic signals. The advantage of SG/OG compared with PGCs is in their easy isolation from testes/ovary to obtain a large number of cells which can be directly microinjected into the body cavity of recipient fry to counteract the potential migration inability of transplanted GC (Yoshikazi et al. 2010). Therefore conservation of GC in LN2 is a valuable tool for maintaining genetic resources. The early stages of germ cells also have the potential for biotechnology such as (1) shortening the reproduction period by using species with a shorter generation interval as hosts; (2) reducing the space required for culture by using small fish as hosts; (3) retaining target species without keeping adult fish; and (4) to acquire knowledge for application in other fish species.

Isolation of germ cells. The efficacy of isolation is facilitated by enzyme-induced dissolution of the germ cells from testicular tissue (Bellve et al. 1977). Lacerda et al. (2006) digested the testes of sexually mature Nile tilapia *Oreochromis niloticus* with collagenase, trypsin, and DNase and isolated the SG from the two upper bands of a Percoll gradient. Spermatogonial stem cells isolated from adult male rainbow trout *Oncorhynchus mykiss* were incubated in PBS (pH 8.2) with 0.5% trypsin (Okutsu et al. 2006b). Psenicka et al. (2012) conducted isolation and cryopreservation of early stages of germ cells of 2–4-year old Siberian sturgeon *Acipenser baerii* with a dissociation medium composed of PBS with 0.2% trypsin and sorted the testicular suspension by Percoll gradient. The highest percentage of GC was obtained from the 10–30% layer of the gradient and confirmed by staining with DDX4. The technique used for tench GC dissociation and isolation was adapted from these studies and optimized for practical use according to relevant data. We recommend using the 5–30% Percoll gradient for tench GC isolation and selection of immature males to obtain a higher concentration of GC compared to mature fish, in which the percentage of early stages of germ cells is lower because of spermatozoa production.

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Cryopreservation. The importance of preserving genetic resources has increased substantially. GC cryopreservation was mainly developed based on information relating to spermatozoa preservation (Billard and Zhang 2001). Successful fish sperm cryopreservation has been achieved by optimizing the freeze/thaw conditions, dilution ratio, freezing and thawing rates, and especially the composition of an extender with cryoprotectants (Suquet et al. 2000; Alavi et al. 2012). A range of cryoprotectants has been tested for fish spermatozoa, with dimethyl sulfoxide, methanol, ethylene glycol, dimethylacetamide, and ethylene glycol being the most commonly used. Significant inter-species variations as well as significant within-species differences in cryoprotectant effectiveness have been reported in fish spermatozoa (Alavi et al. 2012). For example, 8–10% DMSO + P was associated with the highest sperm motility after freeze/thaw in tench (Rodina et al. 2007); MET was reported to be the optimal cryoprotectant for sperm of roach *Rutilus rutilus*, bream *Abramis brama*, silver bream *Blicca bjoerkna*, and barbel *Barbus barbus* (Urbanyi et al. 2006); and DMSO has been recommended for common carp (Horvath et al. 2003). The tested cryoprotectant treatments of tench GC showed no significant differences, indicating high stability of GC in cryoprotectants. Fish GC cryopreservation has been successfully established in rainbow trout (Okutsu et al. 2007) and Siberian sturgeon (Psenicka et al. 2012) under freezing conditions similar to those of the present study. Cryomedium containing 1.8M EG, 0.5% BSA, and 5.5mM D-glucose (Yoshikazi et al. 2011) resulted in a survival rate of GC of rainbow trout after thawing (45.4%) (Okutsu et al. 2007) similar to that obtained with EG in the present study, but lower than when GLY was used (57.69 ± 16.85%). In a study of cryopreservation of GC of Siberian sturgeon Psenicka et al. (2012) found the most effective (61.75% viable) cryoprotectant to be 1.5M EG in extender composed of PBS, 5.5mM D-glucose, and 0.5% BSA. For PGC preservation, an EG concentration of 1.8M resulted in the highest survival rate of thawed PGC (51.3 ± 7.25%) in rainbow trout (Kobayashi et al. 2007). We obtained differing results among individual fish; the male with GC having the highest viability before cryopreservation did not show the highest viability after cryopreservation (Figure 4). Quality of GC within individuals must be taken into account. Another possibility of cryopreservation is to freeze pieces of testes/ovary and dissociate germ cells after thawing (in process) according to Lee et al. (2013). In the future we would like to compare the viability rate of germ cells proceeded by the technique of Lee et al. (2013) with the present study and discuss their effectiveness for practical usage.

CONCLUSION

Isolation and cryopreservation of early stages of germ cells appears to be a useful and simple technique for conservation of genetic resources in tench. The effectiveness of the extender and cryoprotectant is species-specific as well as individually dependent and must be optimized for each fish species. Viability of thawed GC should be verified during each preservation procedure. Our method is simple, it does not require complex laboratory equipment, except for an automatic programmable freezer and swinging head centrifuge for high volume applications. This could be a feasible method for GC isolation and cryopreservation in hatchery practice as well as in research. These data are also considered preliminary results for application in fish bioengineering by transplanting cryopreserved GC into a sterile xenogeneic recipient.

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CHAPTER 5

ISOLATION AND TRANSPLANTATION OF STURGEON EARLY-STAGE GERM CELLS

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Isolation and transplantation of sturgeon early-stage germ cells

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ABSTRACT

We report, for the first time, a series of baseline techniques comprising isolation and transplantation of female and male early-stage germ cells in sturgeon to generate a germline chimera as a potential tool for surrogate reproduction and gene banking. Cells were dissociated from testis, characterized by mostly spermatogonia, and from ovary, exclusively comprising oogonia and previtellogenic oocytes, of *Acipenser baerii*, using 0.3% trypsin (2 hours, 23 °C) dissolved in PBS, isotonic with blood plasma. The dissociated germ cells were sorted by Percoll gradient centrifugation followed by immunolabeling with germ cell–specific vasa antibody DDX4, while 10% to 30% Percoll solution contained 79.4% and 70.8% labeled testicular and ovarian cells. Sorted germ cells were transplanted into a cavity close to a presumptive genital ridge of newly hatched heterospecific *Acipenser ruthenus* larvae with fluorescein isothiocyanate–labeled endogenous primordial germ cells. The transplanted germ cells were randomly distributed in the body cavity through 30-day posttransplantation (dpt). Subsequently, the cells were organized into genital ridges 50 dpt and proliferated 90 dpt. The number of both transplanted and endogenous germ cells significantly increased from 18.1, 22.2, and 29.1 (30 dpt) to 108.5, 90.8, and 118.5 (90 dpt) in ovarian, testicular, and endogenous germ cells, respectively ($P < 0.05$). The efficiency of transplantation was 60% (counted 90 dpt).

1. Introduction

Sturgeons are called living fossils and are believed to have evolved about 200 million years ago [1]. Twenty-seven species of sturgeon are on the Red List of International Union for Conservation of Nature with 63% listed as critically endangered mainly because of overfishing and habitat destruction. Their long reproductive cycle makes population restoration through natural methods difficult. This is further compounded by the present lack of viable cryopreservation methods for sturgeon eggs and embryos because of high yolk content and low membrane permeability.

Primordial germ cells (PGCs) and early stages of oogonia (OG) or spermatogonia (SG) are precursors of gametes that possess the ability to differentiate into eggs and sperm. They undergo proliferation and recombination of genetic information during the process of gametogenesis. These germ cells can be cryopreserved and transplanted for gene banking and surrogate reproduction *via* germline chimera [2–5]. Recently Saito et al. [6] described the origin, migration, and isolation of PGCs in sturgeon. Nevertheless, manipulation of SG and OG seems to be more effective.

We developed a practical technique for isolation of Siberian sturgeon *Acipenser baerii* SG and OG using enzyme dissociation and Percoll solution sorting. The cells were identified using immunolabeling techniques and electron microscopy and transplanted into newly hatched sterlet *Acipenser ruthenus* larvae. This report describes the crucial steps in the production of germline chimera in sturgeon.

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2. Materials and methods

All experimental procedures were approved by the Animal Research Committee of Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic.

2.1. Fish

The Siberian sturgeon were held in a recirculating aquaculture system at a mean temperature of 10 °C. Gonads from three males and three females, aged 4 years, average gonad/body weight 4.6/926 and 4.2/910 g for males and females, were used for all experiments mentioned in the following. Gonads exhibited maturity stage II: containing mostly SG in testes and OG and previtellogenic oocytes in ovaries. A mature testis of sterlet, aged 5 years (gonad/body weight, 36/1120 g), at stage V was used for immunohistochemistry and histologic examination. The stages were used according to Wildhaber et al. [7] (see Supplementary Figs. 1 and 2). Sterlet broodfish were reproduced according to Pšenička et al. [8]. Newly hatched larvae obtained from the mixture of eggs and sperm from three females and three males were used as recipients for isolated gonadal cells.

2.2. Immunoblotting analysis

Proteins were extracted from the gonads and somatic tissue (fin and muscle as control) with a lysis buffer (8M urea, 2M thiourea, 4% CHAPS, 10% wt/vol isopropanol, 0.1% wt/vol Triton X-100, and 100-mM dithiothreithol) containing protease inhibitors (100-mM PMSF, 1 μg mL^{-1} pepstatin A, and 5 μg mL^{-1} leupeptin). The Bradford protein assay [9] was applied to determine protein concentration of the samples. For SDS-PAGE, samples, 25 μg of proteins per lane, were resuspended in a buffer containing 65-mM Tris, 10% (v:v) glycerol, 2% (wt/vol) SDS, and 5% (v:v) β-mercaptoethanol and boiled for 3 minutes at 95 °C. Proteins were separated on 12% SDS gel. After electrophoresis, the gels were placed on polyvinyl difluoride membranes (Bio-Rad, USA) and electrically transferred. The membranes were blocked by incubation with 5% (wt/vol) skim milk in TBST (0.1% Tween-20, 20-mM Tris, 500-mM NaCl at pH 7.6) for 1 hour at 20 °C. The membranes were incubated for 12 hours at 4 °C in 5% BSA-TBST containing DDX4, a rabbit polyclonal antibody (GTX116575, GeneTex), as the primary antibody specific for germline cells and subsequently incubated with Horseradish Peroxidase-conjugated goat antirabbit immunoglobulin G (1:3000 in 3% BSA-TBST) for 1 hour at 20 °C. The reacted proteins were revealed with 3,3′,5,5′-tetramethylbenzidine liquid substrate.

2.3. Immunohistochemistry of gonads

Fragments of gonad and muscle (as control) were fixed in 4% paraformaldehyde in PBS for 1 hour, washed 3 times in PBS for 10 minutes, embedded in 7.5% and subsequently in 15% sucrose for 2.5 hours at 4 °C, and then in 7.5%, 15%, and 20% gelatin for 12 hours at 37 °C. Sections were cut by CM1850 Cryostat (Leica, Germany) at −20 °C and placed on glass microscope slides. The slides were incubated in PBS with 1% BSA and 0.05% Tween 20 (blocking solution) for 40 minutes, then in the DDX4 antibody diluted 300 times with blocking solution 12 hours at 4 °C, washed 3 times in blocking solution for 10 minutes, stained with secondary antibody antirabbit immunoglobulin G–fluorescein isothiocyanate (FITC; F0382, Sigma) diluted 80 times in blocking solution, washed 3 times in PBS, labeled with 4′,6-diamidino-2-phenylindole solution (3 ng mL^{-1}) for 10 minutes, rewashed, mounted with Vectashield, covered with a coverslip, observed under a fluorescence microscope IX83 (Olympus, Japan) equipped with an ORCA R2 camera (Hamamatsu Photonics, Japan), and processed in the CellSens Olympus software. For comparison, the tissues were prepared for paraffin sections as described by Linhartová et al. [10].

2.4. Enzymatic dissociation of gonad cells

To determine optimal conditions for cells dissociation, 1.5 mL of gonad was divided into 15 tubes containing 5 mL of PBS (Sigma–Aldrich P4417), Hank's balanced salt solution (Sigma–Aldrich H6648), or Leibovitz medium (L-15; Sigma–Aldrich L5520) with differing concentrations of trypsin (T; Sigma–Aldrich T1426) and collagenase (C; Sigma–Aldrich C0130): (1) 0.1% T and 0.1% C, (2) 0.3% T, (3) 0.1% T, (4) 0.3% C, or (5) 0.1% C. The tissues were incubated for 2 hours at 23 °C. The optimal temperature for sturgeon cells (20 °C–25 °C) was determined according to Grunow et al. [11] describing culture of sturgeon cells. Osmolality of media was adjusted to that of blood plasma of the fish used (mean, 238 mOsm kg^{-1}), and pH was adjusted to 8. The obtained suspension was filtered with 50-μm filter (Partec, Germany), to collect larger oocytes and debris, and 1% BSA (Sigma–Aldrich A7511) and 40 μg mL^{-1} DNAse (AppliChem A3778) were added. The tubes with gonad cell suspension were centrifuged at 500× *g* for 30 minutes at 4 °C. The pellets were resuspended in 0.3 mL of media without enzymes. The yield and viability of cells obtained from each combination were evaluated by hemocytometer (Bürker's cell counting chamber) and Live/Dead Cell Double Staining Kit (Sigma–Aldrich, 04511), respectively. The number of cells was counted in 20 squares of the hemocytometer in three repetitions under a microscope (Olympus BH2) at × 100 magnification. For the viability test, at least 500 cells per sample were recorded using the Olympus IX83 microscope.

2.5. Purification of germ cells

The suspension of gonad cells was obtained using the dissociation procedure described. The cell suspension was loaded onto a discontinuous Percoll (Sigma–Aldrich P1644) concentration gradient 5%, 10%, 20%, 30%, 40%, and 50% in PBS and centrifuged at 800× *g* for 30 minutes similar to Yoshikawa et al. [12]. Each cell fraction was removed from the gradient and transferred to a tube, diluted in PBS 1:10, and centrifuged again at 800× *g* for 30 minutes. The pellets were resuspended in PBS and examined using immunofluorescence labeling.

2.6. Immunocytochemistry of the sorted cells

The separated gonad cells, with somatic cells from fin and muscle as control, were mounted on poly-l-lysine–coated microscope glass slides and fixed with 4% paraformaldehyde in PBS for 1 hour. The fixed cells were washed in PBS and permeabilized with 0.3% Triton X-100 and subsequently processed as described for immunohistochemistry.

2.7. Transplantation of germ cells

The larvae of sterlet a week after hatching with FITC-labeled endogenous PGCs according to Saito et al. [6] were used as recipients. All together, 120 recipients were analyzed (40 with testicular cells, 40 with ovarian cells, and 40 without transplantation of any cells as control). The enriched gonadal germ cells obtained by Percoll gradient were labeled using the PKH26 Red Fluorescent Cell Linker Kit for general cell membrane labeling according to the manufacturer's protocol (final concentration of dye, 2 μL for 5 minutes), loaded into a glass needle, and injected near the presumptive genital ridge of the host using a micromanipulator M-152 (Narishige, Japan) and a microinjector CellTram Vario (Eppendorf, Germany) under a fluorescent stereomicroscope M165FC. The larvae were kept at 18 °C and fed by *Tubifex*. Ten larvae from each group were examined by the fluorescence stereomicroscope at 6, 30, 50, and 90 days posttransplantation (dpt).

2.8. Statistical analysis

To compare the gain of cells from different dissociation media and the number of endogenous and transplanted cells, two-way ANOVA and Tukey's test using Statistica for Windows, v. 9.1 (StatSoft, Inc., USA) were applied. Probability values of $P < 0.05$ were considered as significant.

3. Results

3.1. Immunoblot analysis with DDX4 antibody

The antibody recognized a single band in all three testicular protein samples of approximately 75 kDa. All three ovarian protein samples showed a double band, suggesting two isoforms of vasa protein in sturgeon females. On the other hand, the control samples of testicular tissue did not show any expression of DDX4 antibody (Supplementary Fig. 2).

3.2. Immunohistochemistry of gonads

Immunohistochemistry showed specific labeling of the early stages of germ cells using DDX4 antibody. The sections of mature testes (stage V) showed specific labeling of SG localized around sperm cysts (Fig. 1A). The testes used for dissociation of cells at maturity stage II contained approximately 1 to 4 SG per cyst (Fig. 1B), whereas the ovaries contained OG with a strong signal and previtellogenic oocytes with a weaker signal (Fig. 1C). Figure 2A, B, C shows paraffin sections of gonads in the same stages for comparison.

3.3. Dissociation of gonad cells

The significantly higher yield of testicular and ovarian cells was obtained after dissociation of tissues in media containing 0.3% trypsin, whereas the significantly lower yield was recorded for media with 0.1% collagenase (Fig. 3A, B; $P < 0.05$).

The viability test did not reveal any significant differences with the exception of lower viability of testicular cells in Hank's balanced salt solution with 0.1% collagenase (Fig. 3C, D; $P < 0.05$).

3.4. Purification of gonad cells

The suspension above 10% of Percoll solution contained mostly only oily droplets. The percentage of DDX4 antibody positive cells collected from 10%, 20%, 30%, 40%, and 50% Percoll solution was 79.6%, 82.4%, 76.3%, 40.5%, and 33.0% for testicular and 83.6%, 74.6%, 54.2%, 21.9%, and 10.4% for ovarian cells, respectively, whereas dissociated control somatic cells showed no fluorescent signal after immunolabeling with DDX4. On the basis of this finding, 10% to 30% Percoll solution was used for the enrichment of germ cells from sturgeon gonad cells (Fig. 4A, B).

A total of 1.35 and 0.93 million DDX4 positive cells in average were isolated from one male and one female, respectively.

3.5. Transplantation of purified germ cells

The donor (Siberian sturgeon) germ cells were labeled, transplanted, and traced in host (sterlet) larvae. The survival rate did not differ between larvae used for transplantation and the control (96.7% and 95.8%, respectively). Six dpt, transplanted cells were observed in 95% of recipient larvae (Table 1, Fig. 5A). The cells floated in the abdominal cavity with some randomly weakly attached to the abdominal wall. Thirty dpt, the cells were randomly tightly attached to the dorsal abdominal wall (Fig. 5B). Fifty dpt, the cells were already organized into genital ridges (Fig. 5C). Ninety dpt, the transplanted cells started proliferation in 60% of recipient fish (Fig. 5D). The Table 1 shows significant increase in average number of PKH26-labeled transplanted as well as FITC-labeled endogenous cells at this time. The control did not show any red-labeled cells at any time of the examination.

4. Discussion

We developed, for the first time, a series of techniques of enzyme dissociation, Percoll concentration gradient sorting and transplantation of sturgeon testicular and ovarian cells. Sterlet, one of the most common and smallest sturgeon species with shortest reproductive cycle (about 5 years), was used as the recipient and Siberian sturgeon, bigger and endangered species with later maturation (18–28 years), as the donor of cells. Because these two species can hybridize with each other [13], it is suggested that they are close related and could be used for the standardization of transplantation experiments.

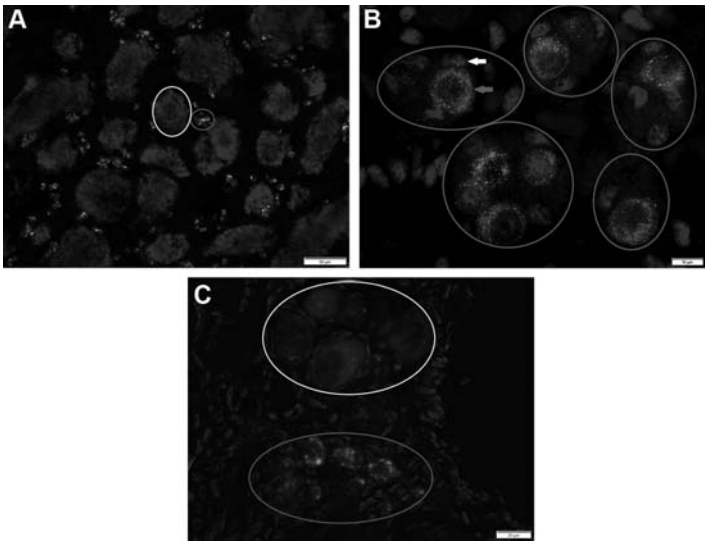

Fig. 1. Immunohistochemistry of sturgeon gonads labeled with DDX4. The figure shows immunohistochemistry of sterlet *Acipenser ruthenus* testis in maturity stage V (5 years old; A, scale bar = 50 μm); and Siberian sturgeon *A baerii* testis at maturity stage II (4 years old; B, scale bar = 10 μm) with cysts containing spermatozoa (white circle) and spermatogonia (red circles and red arrow) surrounded with Sertoli cell (white arrow); and Siberian sturgeon *A baerii* ovary at maturity stage II (4 years old) showing a nests with oogonia and/or early oocytes (red circle) and previtellogenic oocytes (white circle; C, scale bar = 20 μm). Sections were labeled with DDX4 primary antibody conjugated with antirabbit immunogloubulin G–fluorescein isothiocyanate (FITC) secondary antibody and DAPI, whereas FITC (green) specifically labeled germ cells and DAPI (blue) labeled nuclei of all cells. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

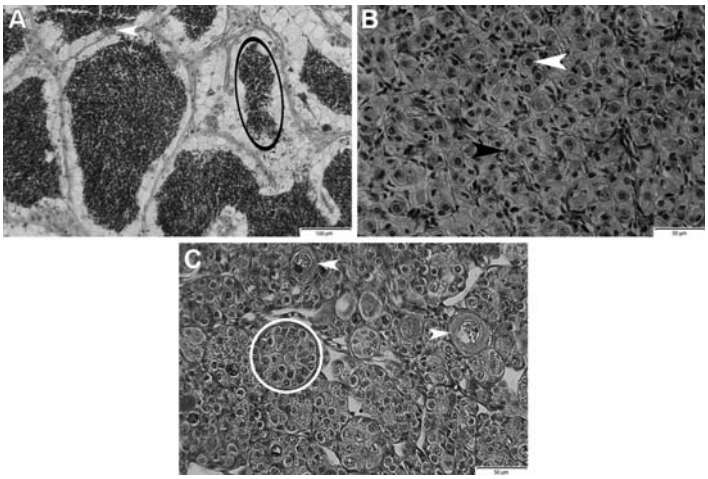

Fig. 2. Histology of sturgeon gonads. The figure shows histology of sterlet *Acipenser ruthenus* testis in maturity stage V (5 years old; A, scale bar = 100 μm); Siberian sturgeon *A baerii* testis at maturity stage II (4 years old; B, scale bar = 50 μm) showing spermatozoa (black ellipse), spermatogonia (white arrow), and Sertoli cell (black arrow); and Siberian sturgeon *A baerii* ovary at maturity stage II (4 years old) showing a nests with oogonia and/or early oocytes (white ellipse) and previtellogenic oocytes (white arrow; C, scale bar = 50 μm). (For interpretation of the references to color in this figure, the reader is referred to the Web version of this article.)

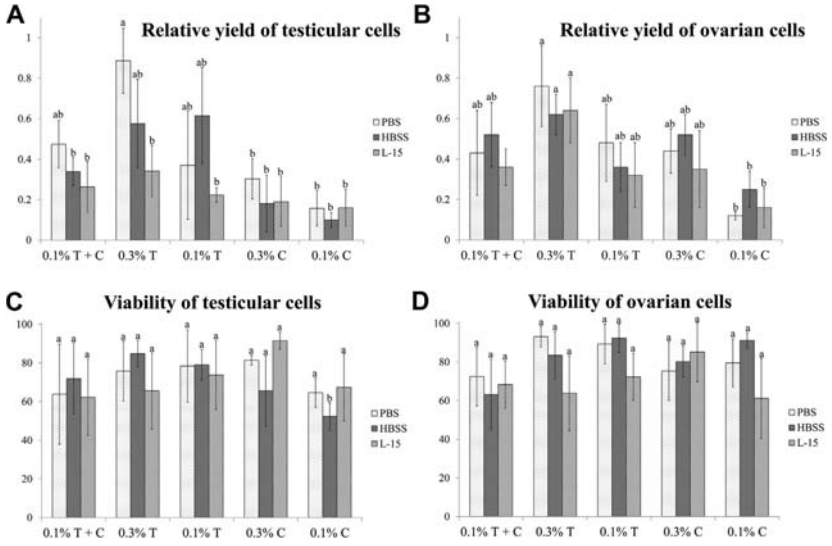

Fig. 3. Graph with relative yield and viability of sturgeon gonadal cells after dissociation. The figure shows mean and standard deviation of relative yield of Siberian sturgeon *Acipenser baerii* testicular (A) and ovarian (B) cells and viability of testicular (C) and ovarian (D) cells (%) after dissociation using different media and concentrations of trypsin and collagenase. Analysis was performed on three samples for each gender at maturity stage II (4 years old). Values with the different superscript letters are significantly different (ANOVA, $P < 0.05$). C, collagenase; HBSS, Hank's balanced salt solution; L-15, Leibovitz medium; T, trypsin.

Brinster and Zimmermann [14] were first to introduce regeneration of spermatogenesis in a mouse model by transplanting SG of the donor into testes of an infertile recipient. Spermatogonia were shown to colonize and develop in recipient mice testes and develop fertile sperm with the donor genotype characteristics. These authors dissociated the SG using 0.1% collagenase and 0.25% trypsin in two steps and used a separation method for stages of mice male germ cells on the basis of sedimentation speed in a concentration gradient of 2% to 4% BSA developed by Bellvé et al. [15]. Okutsu et al. [16] applied this technique to rainbow trout *Oncorhynchus mykiss* for the first time, and Okutsu et al. [17] performed a xenogeneic SG transplant from rainbow trout to the masu salmon *Oncorhynchus masou*. After dissociation of trout testes with 0.5% trypsin in PBS, they showed that, after SG transplantation into the peritoneal cavity, testicular germ cells can colonize in embryonic gonads and produce functional sperm and eggs. The SG of the transgenic donor were identified using a green fluorescent protein driven by the cis elements of the vasa gene. Lacerda et al. [18] dissociated testes of tilapia in the same way as Bellvé [15] and enriched SG from testicular cells by a Percoll gradient and transplanted the obtained cells into urogenital papilla. The isolation and transplantation of OG has been described in trout by Yoshizaki et al. [19] and in zebrafish by Wong et al. [20]. The isolation of zebrafish OG was performed by L-15 containing 0.2% collagenase and 500 IU mL^{-1} dispase in case of trout

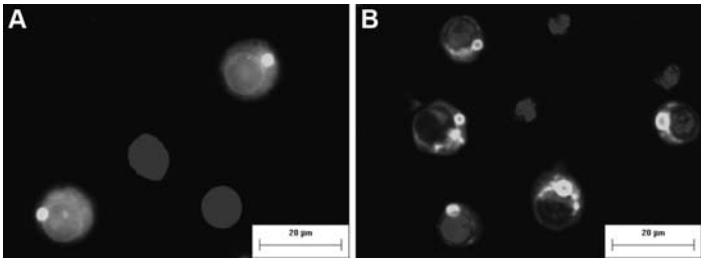

Fig. 4. Immunocytochemistry of sturgeon gonadal cells labeled with DDX4. The figure shows suspension of Siberian sturgeon *Acipenser baerii* testicular (A) and ovarian (B) cells labeled with DDX4 primary antibody conjugated with antirabbit immunogloubulin G–fluorescein isothiocyanate (FITC) secondary antibody and DAPI, whereas FITC (green) specifically labeled germ cells, and DAPI (blue) labeled nuclei of all cells (scale bar = 20 µm). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

Table 1
The success of sturgeon germ cells transplantation.

Time post-transplantation	Ovarian cells		Testicular cells		Endogenous germ cells
	Positive	Number of cells, mean ± SD	Positive	Number of cells, mean ± SD	Number of cells, mean ± SD
6 dpt	9/10	?	10/10	?	?
30 dpt	7/10	$18.1^{a} \pm 12.1$	8/10	$22.2^{a} \pm 10.8$	$29.1^{a} \pm 16.3$
50 dpt	7/10	$19.8^{a} \pm 9.8$	6/10	$17.1^{a} \pm 8.2$	$25.8^{a} \pm 11.2$
90 dpt	6/10	$108.5^{b} \pm 55.4$	6/10	$90.8^{b} \pm 38.2$	$118.5^{b} \pm 48.4$

Table shows the success of transplantation and average number of transplanted and endogenous germ 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 posttransplantation; SD, standard deviation.

and by PBS containing 0.2% collagenase in zebrafish. The manipulation with OG should be especially considered in sturgeon because sturgeon are suggested to have a female heterogamety (ZW female/ZZ male) sex-determination system [21,22]. Due to this, conservation based only on male germ cells may not be effective because they may not include all genetic information. The authors always used the optimal temperature of the species for enzymatic cells dissociation and balance a lower temperature with a longer exposure time (zebrafish 28.5 °C for 1 hour and trout 10 °C for 7–9 hours). In this study on sturgeon, results showed the use 0.3% trypsin in PBS at 23 °C for dissociation of testicular and ovarian cells to be optimal, as this medium dissociated the highest number of cells without decreasing viability. On the other hand, the dissociation using collagenase was found as statistically worst. Nowadays scientists avoid treatment of stem cell with trypsin. Recently Shikina et al. [23] revealed a disruption of membrane proteins of rainbow trout SG by incubation in trypsin, which causes reversible decreasing of cells mitotic activity. They suggested a recovery of SG *via* short-term *in vitro* cultivation. In this study, the incubation with trypsin came out as the most efficient for dissociation, and the testicular and ovarian cells could proliferate in gonads of recipient after transplantation similarly to the endogenous cells. It signifies that the cells could be at least partially recovered also after cultivation in body cavity of the recipient. Nevertheless, other studies are required regarding the effect of trypsin treatment on sturgeon SG, their possible recovery *in vitro* and other transplantation experiments.

Enrichment of early stages of germ cells was successfully achieved by sorting with 10% to 30% Percoll solution centrifugation in this study, although the number of remaining somatic cells was still quite high. A promising approach was described by Von Schönfeldt et al. [24]. They performed the enrichment of SG by magnetic-activated cell sorting using surface markers of spermatogonial stem cells in several mammalian species. Panda et al. [25] introduced this technique in a cyprinid fish *Labeo rohita*. Nevertheless, it is currently not possible to identify germ stem cells in sturgeons because of lack of a germ stem cell–specific marker. In this study, the isolated germ cells were identified by their expression of vasa protein, which is a conserved germline marker in animals. Recently Rzepkowska and Ostaszewska [26] studied early gonad development of cultured Siberian and Russian sturgeon on the basis of vasa antibody. They identified vasa protein in germline cells. In the present study, immunoblot analysis clearly exhibited double bands in ovarian protein and a single band in testicular protein analyses with DDX4 antibody, suggesting a sex-linked differential expression of vasa splice variants as previously described in zebrafish by Krøvel and Olsen

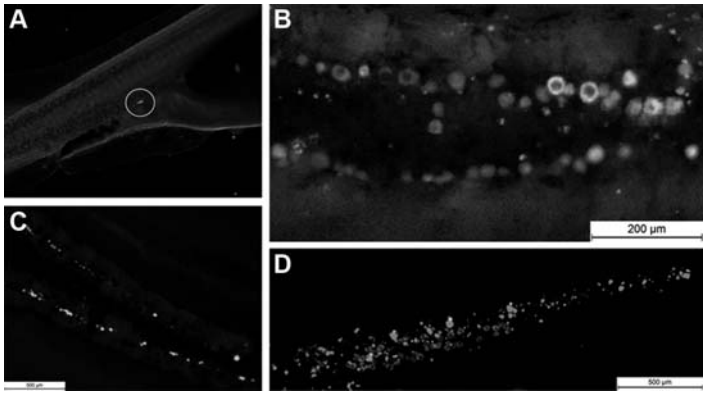

Fig. 5. Sterlet *Acipenser ruthenus* genital ridge with transplanted Siberian sturgeon *Acipenser baerii* germ cells. Sterlet *A ruthenus* with fluorescein isothiocyanate–labeled (green) endogenous primordial germ cells and PKH26-labeled (red) transplanted gonadal cells of Siberian sturgeon *A baerii*: (A) 6 days post-transplantation (dpt) with spermatogonia (SG) in a living larva (scale bar = 1 mm), (B) 30 and (C) 50 dpt with transplanted SG (scale bar = 200 and 500 µm), and (D) 90 dpt with transplanted oogonia (scale bar = 500 µm) after dissection.

[27]. These proteins could be used in aquaculture for early sexing of sturgeon.

Rzepkowska and Ostaszewska [26] have shown that the beginning of proliferation and differentiation in Siberian and Russian sturgeon occurs around 115 days after hatching. In the present study, the increased number of FITC-labeled endogenous sterlet gem cells and PKH26-labeled transplanted Siberian sturgeon cells were observed 90 dpt (97 days after hatching). Nevertheless, we admin that simple labeling is not a proper tool to identify interspecific germline chimera because of a possible contamination with somatic cells, and development of a germline species-specific genetic marker is required in future studies.

Another possible technique to create a germline chimera is the transplantation of PGCs, but the number of visualized PGCs from a single sturgeon embryo is only 23.5 on average [6], and transplantation techniques using PGCs are not promising from the standpoint of efficiency. Spermatogonia and OG are considered suitable alternatives to PGC transplantation because the yield from a single individual can be large. The yield of DDX4 positive cells from an individual sturgeon in our trial was approximately one million. This high amount of cells could be achieved because of relatively large size of gonads with late sexual maturity of sturgeon. For comparison, Wong et al. [20] isolated 755 vasa positive ovarian cells from one zebrafish.

For future studies of surrogate reproduction *via* germline chimerism, a sterile host is required. To sterilize the sturgeon host, a reversible knockdown of dead-end gene using antisense morpholino oligonucleotide is a promising technique [28]. This approach could be a powerful tool in conservation genetics of this endangered group of species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2014.12.010.

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CHAPTER 6

STERILIZATION OF STERLET *ACIPENSER RUTHENUS* BY USING KNOCK DOWN AGENT, ANTISENSE MORPHOLINO OLIGONUCLEOTIDE, AGAINST *DEAD END* GENE

Linhartova, Z., Saito, T., Kaspar, V., Rodina, M. Hagihara, S., Psenicka M., 2015. Sterilization of sterlet *Acipenser ruthenus* by using knock down agent, antisense morpholino oligonucleotide, against *dead end* gene. Theriogenology: doi: 10.1016/j.theriogenology.2015.07.003.

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Sterilization of sterlet *Acipenser ruthenus* by using knockdown agent, antisense morpholino oligonucleotide, against *dead end* gene

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ABSTRACT

Sturgeons (chondrostean, acipenseridae) are ancient fish species, widely known for their caviar. Nowadays, most of them are critically endangered. The sterlet (*Acipenser ruthenus*) is a common Eurasian sturgeon species with a small body size and the fastest reproductive cycle among sturgeons. Such species can be used as a host for surrogate production; application is of value for recovery of critically endangered and huge sturgeon species with an extremely long reproductive cycle. One prerequisite for production of the donor's gametes only is to have a sterile host. Commonly used sterilization techniques in fishes such as triploidization or hybridization do not guarantee sterility in sturgeon. Alternatively, sterilization can be achieved by using a temporary germ cell exclusion–specific gene by a knockdown agent, the antisense morpholino oligonucleotide (MO). The targeted gene for the MO is the *dead end* gene (*dnd*) which is a vertebrate-specific gene encoding a RNA-binding protein which is crucial for migration and survival of primordial germ cells (PGCs). For this purpose, a *dnd* homologue of Russian sturgeon (*Agdnd*), resulting in the same sequence in the start codon region with isolated fragments of sterlet *dnd* (*Ardnd*), was used. Reverse transcription polymerase chain reaction confirmed tissue-specific expression of *Ardnd* only in the gonads of both sexes. *Dnd*-MO for depletion of PGCs together with fluorescein isothiocyanate (FITC)–biotin–dextran for PGCs labeling was injected into the vegetal region of one- to four-cell-stage sterlet embryos. In the control groups, only FITC was injected to validate the injection method and labeling of PGCs. After optimization of MO concentration together with volume injection, 250-μM MO was applied for sterilization of sturgeon embryos. Primordial germ cells were detected under a fluorescent stereomicroscope in the genital ridge of the FITC-labeled control group only, whereas no PGCs were present in the body cavities of morphants at 21 days after fertilization. Moreover, the body cavities of MO-treated and nontreated fish were examined by histology and *in situ* hybridization, showing gonads which had no germ cells in morphants at various stages (60, 150, and 210 days after fertilization). Taken together, these results report the first known and functional method of sturgeon sterilization.

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1. Introduction

With the recent worldwide poaching of sturgeons for caviar, construction of dams, and environmental pollution, these fishes are listed in the International Union for the Conservation of Nature's Red List as the most endangered group of species in the world. Moreover, their artificial reproduction is complicated by late maturation and the inability of females to repeat reproduction every year [1]. Therefore, an efficient approach for their reproduction and conservation is needed. In recent years, techniques for surrogate production through germ line chimera by early germ cells transplantation into closely related species have been established in several teleostean species [2,3]. The sterlet (*Acipenser ruthenus*) is one of the most common and smallest Eurasian sturgeon species with the fastest reproductive cycle (sexual maturity of males 3–7 and females 5–9 years of age) in comparison with other sturgeons [1]. A prerequisite to generate germ line chimera, producing the donor's gametes only, is the sterilization of the host. Triploidization is the method of choice for practical use in species whose triploid individuals are sterile [4,5]. However, in sturgeons (evolutionary polyploids), all ploidy levels are probably fertile [6]; therefore, an alternative technique for sturgeon sterilization must be considered. The approach was to use a temporary exclusion of the germ cell–specific gene that is responsible for their development by a knockdown agent, from which the antisense morpholino oligonucleotide (MO) was applied. *Dead end* (*dnd*), a gene that encodes an RNA-binding protein crucial for migration and survival of primordial germ cells (PGCs), was selected. This gene has already been described in a number of model species, and knockdown of *dnd* by the MO interferes with PGC migration and results in their death [7,8].

2. Materials and methods

2.1. Ethics

All experiments were carried out in accordance with the Animal Research Committee of the Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Fish were maintained according to the principles based on the EU harmonized animal welfare act of Czech Republic, and principles of laboratory animal care and the national laws 246/1992 "Animal welfare" on the protection of animals were followed and respected.

2.2. Fish source, embryos preparation, and sample collection

Adult sterlet (*A ruthenus*) females and males, aged 5 to 9 years, were transferred from outdoor ponds into indoor recirculating aquaculture system during the spawning season March to June 2014. Fish were held in 4000-L tanks at mean water temperature of 13 °C. To induce spermiation, males were injected by a single intramuscular injection of carp pituitary extract at 4 mg/kg of body weight (BW) in 0.9% NaCl. Sperm was collected 48 hours after hormone injection and kept on ice at 4 °C until fertilization. Spermatozoa motility was assessed by light microscopy and was greater than 90%. Ovulation was stimulated with carp pituitary extract by intramuscular injection in two doses: the first dose, 0.5 mg/kg of BW and the second, 4.5 mg/kg of BW, 12 hours after the first injection. The ovulated eggs were collected from three females 18 to 20 hours after the second injection. The eggs were inseminated with sperm from two males in dechlorinated water at 15 °C. Stickiness of the fertilized eggs was removed by treating with 0.04% tannic acid. Eggs were dechorionated (outer layer) 1 hour after fertilization using forceps. Dechorionated eggs were transferred to 100-mL dechlorinated tap water with 0.01% penicillin and streptomycin in glass Petri dishes and incubated at 15 °C in an incubator. Temperature was regulated at 15 ± 1 °C throughout the experiment, and water was changed daily. Embryos were mainly used for injection of fluorescein isothiocyanate (FITC)–biotin–dextran for PGCs labeling, antisense MO for PGCs depletion, reverse transcription polymerase chain reaction (RT-PCR), histology, and *in situ* hybridization (ISH, described in the following).

Tissue samples used for determination of *dnd* gene expression by RT-PCR were obtained from adult sterlet males and females; they were rapidly dissected and washed in PBS (adjusted to 248 mOsm/kg, pH 8). For RNA extraction, tissues were frozen in liquid nitrogen and stored at −80 °C.

2.3. Isolation of Ardnd fragments (sterlet dnd) for comparison with full-length Agdnd (Russian dnd)

The *dnd* gene of Russian sturgeon (*Agdnd*; Hagihara, unpublished data) was used as a reference sequence for MO design. Full length of Russian *dnd* (*Agdnd*) was homology searched and identified from the database of transcriptome in a developing gonad by Hagihara (unpublished data). For MO design, the sequence of gene in the start codon region has to be known; therefore, only fragments of sterlet *dnd* (*Ardnd*) including the ATG region were identified in this study and compared with *Agdnd*. For this purpose, total RNA was isolated from stripped unfertilized eggs of sterlet using RNeasy Lipid Tissue Mini Kit (Qiagen, Prague, Czech Republic). First-strand complementary DNA (cDNA) was synthesized from 1 to 4 μg of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany). The cDNA fragments of *Ardnd* gene were amplified by RT-PCR using primers (Table 1) designed according to the full-length sequence of *Agdnd* (Hagihara, unpublished data). Polymerase chain reaction was performed using a C1000 thermal cycle (Bio-Rad, Hercules, CA, USA) under following conditions: denaturation at 95 °C for 5 minutes, 35 cycles of amplification at 95 °C for 20 seconds, 60 °C for 30 seconds, 72 °C for 1 minute, and additional elongation at 72 °C for 10 minutes. Polymerase chain reaction products were separated by 1.2% agarose gel electrophoresis. The fragments were purified by NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany) and cloned into pCR 2.1-TOPO TA vector using TOPO TA Cloning Kit with competent *Escherichia coli* cells (Life Technologies, Prague, Czech Republic). Positive clones were amplified by PCR as follows: initial step at 95 °C for 2 minutes, 5 cycles at 95 °C for 30 seconds, 50 °C for 1 minute, 72 °C for 1 minute, followed by 30 cycles at 95 °C for 30 seconds, 50 °C for 45 seconds, 72 °C for 1 minute, and a final

Table 1
Ardnd gene–specific primers used for isolation of *Ardnd* fragments and reverse transcription polymerase chain reaction analysis.

Primer name	Forward sequences (5′–3′)	Reverse sequences (5′–3′)	Size (bp)
Ardnd1	AAACGTGAGGCACGGGTATT	CCTGGATCGGTATCCACAGC	705
Ardnd2	TGCGGCGCTCTCAAAGTAAT	TGAACTCATAGAGCACGCCC	47
Ardnd3	AGTTCCTGTTGACACGCTCC	ATACCCGTGCCTCACGTTTT	89
Ardnd4	AGCTGGCTGTGGATACCGAT	TGAACATGCAAGACAAAAAGTGAGT	786
CytB	TATTCATGCAAACGGGGCCT	ACGGTTGCCCCTCAAAATGA	192

extension at 72 °C for 20 minutes and sequenced by Macrogen company (Macrogen Inc., Amsterdam, the Netherlands).

2.4. Sequence analysis and alignment

Sequenced fragments of *Ardnd* were aligned with provided data of full-length *Agdnd* (to find the ATG region) using the BioEdit software (BioEdit version 7.1.3.0, 2011). Moreover, homology and identity searches of *Agdnd* with *dead end* homologue of the Chinese sturgeon *Acipenser sinensis* (*Asdnd*, KM 655832.1, [9]) (the only published *dnd* in acipenseridae family) were performed using the online Web site (http://www.ncbi.nlm.nih.gov/gene).

2.5. Construction of sterlet/Russian sturgeon dnd morpholino (dnd-MO)

To perform sterilization of sterlet/Russian sturgeon by *dnd* gene knockdown, an antisense MO was designed in conserved region including start codon by Gene Tools (Gene Tools, LLC, Philomath, OR, USA; Table 2).

2.6. Microinjection of dnd-MO

Dehydrated 2-mM *dnd*-MO was resuspended in nuclease-free water with 0.2-M KCl. To label PGCs, 1% FITC–biotin–dextran (molecular weight = 500,000) was coinjected with *dnd*-MO according to Saito et al. [10]. In the control groups, only 1% FITC was injected. Moreover, a commercially available *dnd*-MO for zebrafish with 1% FITC was also tested as a control to guarantee the specificity of sterlet *dnd*-MO. A glass micropipette was drawn from a glass needle (Drummond, Tokyo, Japan) using a needle puller (PC-10; Narishige, Tokyo, Japan). The *dnd*-MO + FITC or just FITC was loaded into the micropipette and injected into the vegetal pole of sterlet embryos at the one- to four-cell-stage at 1 to 4 hours after fertilization. Microinjection was performed under a fluorescent stereomicroscope Leica M165 FC (Leica, Wetzlar, Germany) using a micromanipulator M-152 (Narishige) and microinjector FemtoJet express microinjector (Eppendorf, Hamburg, Germany) with a pressure of 100 hPa for 1 second. To determine the optimal dose for PGCs depletion, ten eggs from each female (three individuals) were injected with different concentrations of *dnd*-MO (100, 250, 500, 750, and 1000 μM) and survival rate and numbers of FITC-labeled PGCs at 4 days post fertilization (dpf) were examined. At 21 dpf, larvae from each group were anesthetized by tricaine solution, the body cavity was opened; the gut was dissected, positions of PGCs were checked, and they were counted. After selection of best concentration of *dnd*-MO (250 μM), 300 embryos of two females were selected, in which 100 were used for injection of 250-μM *dnd*-MO + FITC, the same number were used as the control group by injection of FITC, and the remaining embryos were kept as noninjected controls for further incubation to assess developmental rate. The embryos were kept at 15 °C, and hatched larvae were fed with Tubifex. Development of embryos was documented by the stereomicroscope Leica M165 FC with a camera (Leica DFC425C), and the numbers of positive fish with labeled FITC-PGCs were counted at 4 dpf. Larvae of each group were separately transferred into 55-L aquariums and kept for histologic, RT-PCR, and ISH analyses.

Table 2
Sequence of *Agdnd* used for MO design and the designed sequence of *dnd*-MO.

Gene/MO	Region	Sequences (5′–3′)	Size (bp)
Agdnd	161–214	CAAGTTGTCTGGAGAAAACATCTTAAGAA [AGAAA(ATG)ATTGAAGGAGAGCAAAC]	53
dnd-MO	—	CCTCTGTTTGCTCTCCTTCAATCAT	25

Brackets, [], show the position of *dnd*-MO in *Agdnd* and, (), the presence of start codon.
Abbreviation: MO, morpholino oligonucleotide.

2.7. RT-PCR analysis

Reverse transcription polymerase chain reaction was used to detect expression of the *Ardnd* gene in several tissues of two adult sterlet males and females to confirm the hypothesis that *Ardnd* is a germ cell–specific gene expressed in gonads only. Moreover, gonads of five MO-treated fish and five control specimens at 210 dpf were used for the analyses. Total RNA extraction and

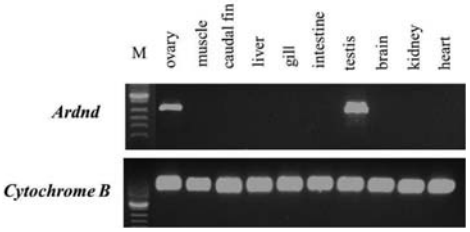

Fig. 1. Tissue-specific expression of *Ardnd* and *Cytochrome B* determined by reverse transcription polymerase chain reaction. M, reference marker.

Table 3
Test of different concentrations of *dnd*-MO (coinjected with FITC) on the survival rate and number of FITC-labeled PGCs compared with control FITC-injected and noninjected fish at 4 and 21 days post fertilization (dpf).

Experimental group	Total no. of embryos	Survival rate (%) after hatching	No. (mean ± SD) of FITC-PGCs at 4 dpf	No. (mean ± SD) of FITC-PGCs at 21 dpf
dnd-MO 1000	30	20.0[d]	0[c]	0[c]
dnd-MO 750	30	20.0[d]	0[c]	0[c]
dnd-MO 500	30	40.0[c]	0[c]	0[c]
dnd-MO 250	30	63.3[b]	0.1 ± 0.45[c]	0[c]
dnd-MO 100	30	73.3[a]	4.65 ± 4.92[b]	3.86 ± 3.44[b]
Control FITC	30	63.3[b]	15.42 ± 8.01[a]	13.29 ± 7.59[a]
Control noninjected	30	63.3[b]	—	—

Values with the same character in each column are not significantly different (one-sample *t* test, $P < 0.05$).
Abbreviations: FITC, fluorescein isothiocyanate; MO, morpholino oligonucleotide; PGCs, primordial germ cells; SD, standard deviation.

transcription to cDNA were performed as described previously using 50 to 100 mg of various tissues (ovary, testes, caudal fin, liver, gill, intestine, muscle, brain, kidney, and heart). Reverse transcription polymerase chain reaction was performed using the *Ardnd1*-specific primers (Table 1). *Cytochrome B (CytB)*, a housekeeping gene, was amplified as a control that had to be expressed in all analyzed tissues, by the primers *CytB* F and R (Table 1). Reverse transcription polymerase chain reaction was run with a reaction volume of 20 μL containing 20 to 30 ng of cDNA under following conditions: 95 °C for 5 minutes, 35 cycles of amplification at 95 °C for 20 seconds, 60 °C for 30 seconds, 72 °C for 1 minute, and final extension at 72 °C for 10 minutes.

2.8. Histologic analysis

Ten fish at 60 dpf, five fish at 150 dpf, and five fish at 210 dpf of *dnd*-MO (250 μM)–injected and control (noninjected) groups were sacrificed; their trunks (at 60 dpf) or gonads (from 60 dpf) were dissected and washed in PBS. Gonads or dissected trunks of fish were fixed in Bouin's solution overnight and stored in 80% ethanol until further processing. Then, samples were dehydrated in an ethanol–xylene series, embedded in paraffin blocks, cut into 5-μm-thick sections using a rotary microtome Diapath (Diapath Galileo, Italy). Paraffin slides were stained with hematoxylin and eosin by using a staining machine (Tissue-Tek DRS 2000; Sakura, Torrance, CA, USA) according to standard procedures. The state of gonads was evaluated from histologic sections by the optical microscope Olympus BH2 (Olympus Corp., Tokyo, Japan) at × 200 and × 400 magnification and photographed by the Nikon D5100 camera (Nikon, Tokyo, Japan).

Table 4
Survival rate and percentage of fish with labeled FITC-PGCs in experimental and control groups at 4 days post fertilization (dpf).

Experimental group	Total no. of embryos	Survival rate (%) after hatching	Percentage of embryos with FITC-PGCs at 4 dpf
dnd-MO 250	100	88.2[b]	4.9[b]
Control FITC	100	81.4[b]	82.5[a]
Control noninjected	100	92[a]	0[b]

Values with the same character in each column are not significantly different (one-sample *t* test, $P < 0.05$).
Abbreviations: FITC, fluorescein isothiocyanate; MO, morpholino oligonucleotide; PGCs, primordial germ cells.

2.9. In situ hybridization

To detect the expression patterns of *Ardnd*, gonads of five 210-dpf control fish and morphants (same used for histology), ISH was carried out. Also, *vasa* gene, as a germ cell–specific gene, was tested as *Ardnd*. Gonads were fixed in 4% paraformaldehyde overnight and stored in methanol at −20 °C until usage. Sense and antisense *Ardnd* and *vasa* probes were synthesized by PCR using primers with introduced SP6/T7-promoter site. The cDNA sterlet fragments were purified by NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Antisense (positive control) and sense (negative control) digoxigenin-labeled RNA probes were applied using 1-μg cDNA fragment and a DIG RNA Labeling Kit (SP6/T7; Roche) by following the manufacturer's instruction. *In situ* hybridization procedure was established according to Saito et al. [11] and Lin et al. [12]. Photographs were taken using an Olympus stereomicroscope IX83 (Olympus Corp.) equipped with an ORCA R2 camera (Hamamatsu Photonics, Tokyo, Japan).

2.10. Statistical analysis

The percentage of control and *dnd*-MO–treated fish containing FITC-labeled PGCs was analyzed by the one-sample *t* test. Probability values of $P < 0.05$ were considered significant. All statistical analyses were performed using programming language R software (Version 3.0.2, 2014) for statistical analysis of data for MS Windows.

3. Results

3.1. Characterization of sequence and alignment analysis of dnd genes

The full-length Russian *Agdnd* sequence was obtained by next-generation sequencing. *Agdnd* cDNA was 1710-bp long, containing 1290-bp open reading frame, which encoded 430 amino acids. Fragments of *Ardnd* gene were sequenced with specific primers (Table 1) and aligned with *Agdnd* (Supplementary Fig. 1) which resulted in 97% identity. Similar fragments with ATG region in both *dnd* sequences were identified, resulting in one *dnd*-MO design for both species (sterlet and Russian sturgeon).

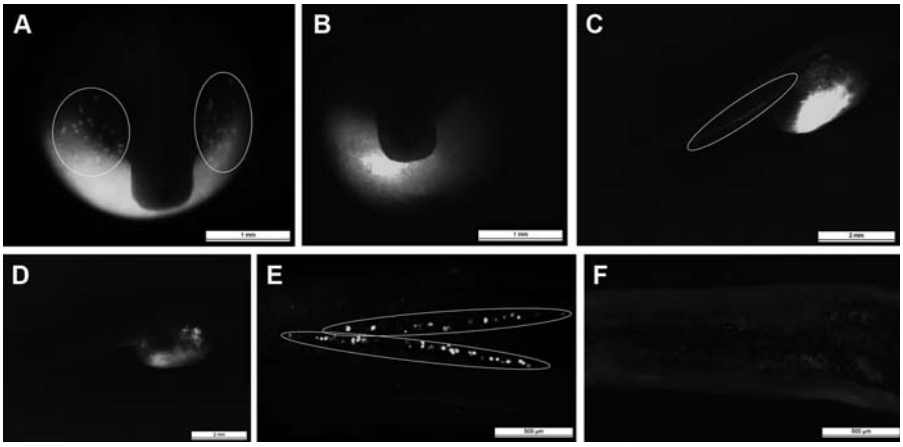

Fig. 2. Ablation of primordial germ cells (PGCs) in sterlet embryos and larvae treated with *dnd*-MO (250 μM) at 4, 7, and 21 days post fertilization (dpf) compared with controls. (A, B) Tail-bud stage, 4 dpf; (C, D) hatching period, 7 dpf; (E, F) dissection of body cavity, 21 dpf. (A, C, E) Control fluorescein isothiocyanate–injected group with PGCs (white circles); (B, D, F) 250-μM *dnd*-MO–injected group. Scale bars are (A, B, E) 1 mm; (C, D) 2 mm, and (E) 500 μm. MO, morpholino oligonucleotide.

3.2. Ardnd expression in different tissues

Reverse transcription polymerase chain reaction showed the tissue-specific expression of *Ardnd* only in gonads, with a higher level of expression in testes. In other tissues, including caudal fin, liver, gill, intestine, muscle, brain, kidney, and heart, *Ardnd* was not detected. *CytB* was used as a reference to determine the distribution pattern in all analyzed tissues (Fig. 1).

3.3. Ardnd knockdown by dnd-MO injection leading to PGC depletion

Dnd-MO was designed and constructed (as previously described, Table 2) to sterilize sterlet by *Ardnd* knockdown. In the first experiment, several *dnd*-MO concentrations were tested (100–1000 μM) and a dose-dependent effect was observed. *Dnd*-MO concentration of 250 μM resulted in 63.3% hatched embryos with average number of visualized

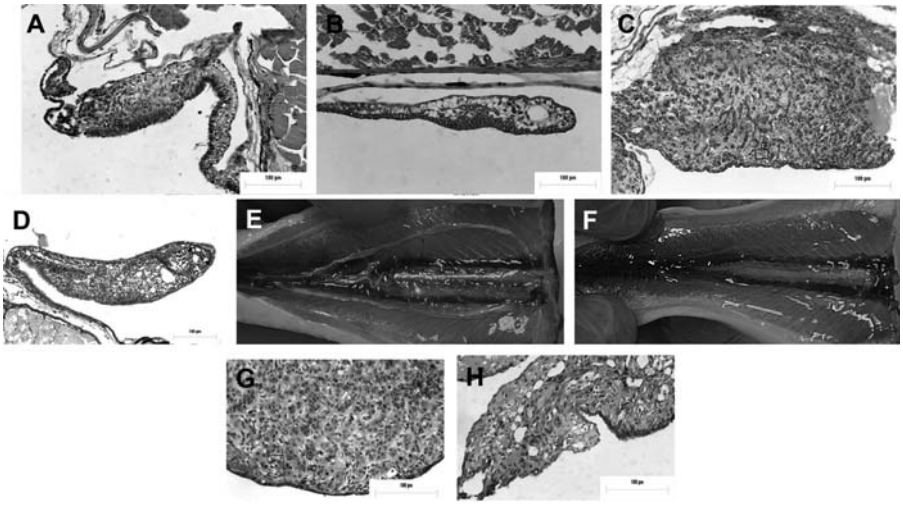

Fig. 3. Occurrence or nonoccurrence of germ cells in transverse histologic sections of sterlet genital ridge at 60, 150, and 210 days post fertilization (dpf) and external appearance of gonads of sterlet at 210 dpf. Hematoxylin–eosin stained transverse 5-μm sections show presence of germ cells (circles) and somatic cells (rectangles) in control noninjected groups and empty spaces in 250-μM *dnd*-MO–treated groups (scale bar = 100 μm). (A, C, E, G) Control noninjected group; (B, D, F, H) 250-μM *dnd*-MO–injected group. MO, morpholino oligonucleotide.

FITC-PGCs, 0.1 ± 0.45. The same hatching rate (63.3%) was observed in the control FITC-injected groups with average number of labeled FITC-PGCs, 15.42 ± 8.01. Therefore, the concentration 250 μM of *dnd*-MO, which led to a significant developmental arrest of PGCs, was used in subsequent experiments. Abnormal development of embryos and low survival rates (40%–20%) of hatched larvae were associated with higher *dnd*-MO concentrations. The lowest concentration of 100-μM *dnd*-MO resulted in incomplete elimination of migrating PGCs (Table 3). Moreover, a commercially available *dnd*-MO for zebrafish was tested and resulted in the same survival rate and number of visualized PGCs comparable with the control FITC-injected groups. This guaranteed that the effect of *dnd*-MO designed for sterlet is specific.

For the second fertility or sterility experiment, 300 developing embryos were selected, 100 were injected with 250-μM *dnd*-MO + FITC, 100 with FITC as a control, and the remaining 100 were kept as noninjected control to assess the developmental rate. No significant differences were observed in survival among FITC controls (81.4% developed) and 250-μM *dnd*-MO–injected fish (88.2%; Table 4). The PGCs in the control FITC-injected embryos were observed at 4 dpf, (Fig. 2A), and almost, no PGCs were detected in the *dnd*-MO group (Fig. 2B, Table 4). After hatching (7 dpf), PGCs migrated dorsally to the upper part of the gut, where the genital ridge is localized (Fig. 2C), whereas no PGCs were observed in morphants (Fig. 2D). At 21 dpf, larvae from each group were checked and PGCs were observed only in the body cavity of FITC-injected individuals. They colonized the positions where the genital ridge is assumed to be localized (Fig. 2E). No PGCs were detected in dissected body cavities of morphants (Fig. 2F). Moreover, *dnd*-MO was tested in Russian sturgeon embryos because *dnd*-MO design was based on the sequence of *Agdnd*, and it affected the development of PGCs in the same way as described in sterlet (data not shown).

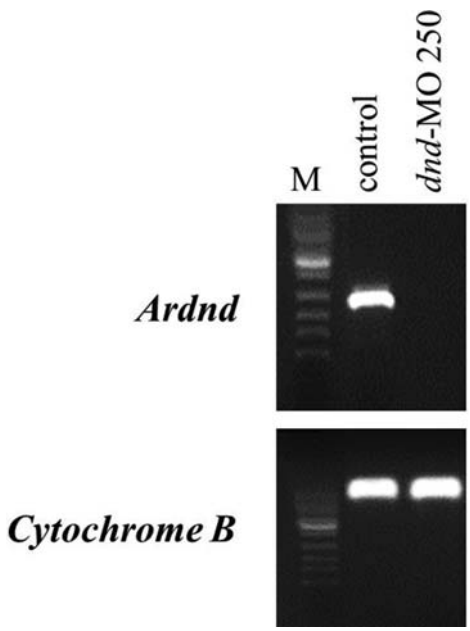

Fig. 4. *Ardnd* and *Cytochrome B* expressions in gonads of 250-μM *dnd*-morpholino oligonucleotide–injected fish and noninjected control fish at 210 days after fertilization determined by reverse transcription polymerase chain reaction. M, reference marker.

3.4. Histologic analysis of morphants and controls during development

Gonadal development was observed in histologic preparations to examine the inner structure of gonads and localization of germ cells. In 60-dpf fish, the ribbon-like gonadal ridges were elongated ventrally from the ventral end of the kidney to caudal positions; they were transparent and poorly visible. In the control groups, the undifferentiated gonadal ridges were covered by notched epithelium and filled with visible layers of PGCs. Primordial germ cells of irregular shape were surrounded by somatic cells (Fig. 3A). On the other hand, the gonadal ridges of morphants contained empty spaces in the genital cavity (Fig. 3B). In 150-dpf juveniles, the gonads of controls were clearly visible, light, and flat in comparison with morphants, in which the external appearance of gonads was comparable with the previous stage (poorly visible). In controls, the gonadal area was occupied by germinal cells (putative late PGCs) and covered with epithelium extending to notches and folds. The gonadal side consisted of fibrous connective tissue with small somatic cells and fat (Fig. 3C). In morphants, the holes in "partially empty" gonads increased in size and were occupied by a large number of blood vessels and somatic cells (Fig. 3D). In 210-dpf juveniles, the gonads were elongated in the whole length of the body cavity. The external appearance of gonads of controls and morphants was significantly different: light gonads of lamellar or flat structure of controls (Fig. 3E) and narrow, transparent or light, tube-like gonads of morphants (Fig. 3F). In the control group, the gonads consisted of large numbers of germ cells that started to proliferate into later stages of germ cells (Fig. 3G). On the other hand, gonads of morphants consisted of only blood vessels and somatic cells, without any germ cells. Visible "holes" were formed in the positions of missing germ cells (Fig. 3H).

3.5. RT-PCR and ISH analysis of gonads of juvenile morphants and controls

Moreover, a part of gonad of five juveniles (210 dpf) of each group was examined by RT-PCR and ISH to determine the expression level of *Ardnd* and *vasa* and localization. Reverse transcription polymerase chain reaction showed that *Ardnd* is expressed only in products of control fish, and no signal was detectable in morphants (Fig. 4). *CytB* was used as a reference to determine the positive distribution pattern in gonads. A positive signal restricted to germ cells was detected by ISH with *Ardnd* and *vasa* antisense probes

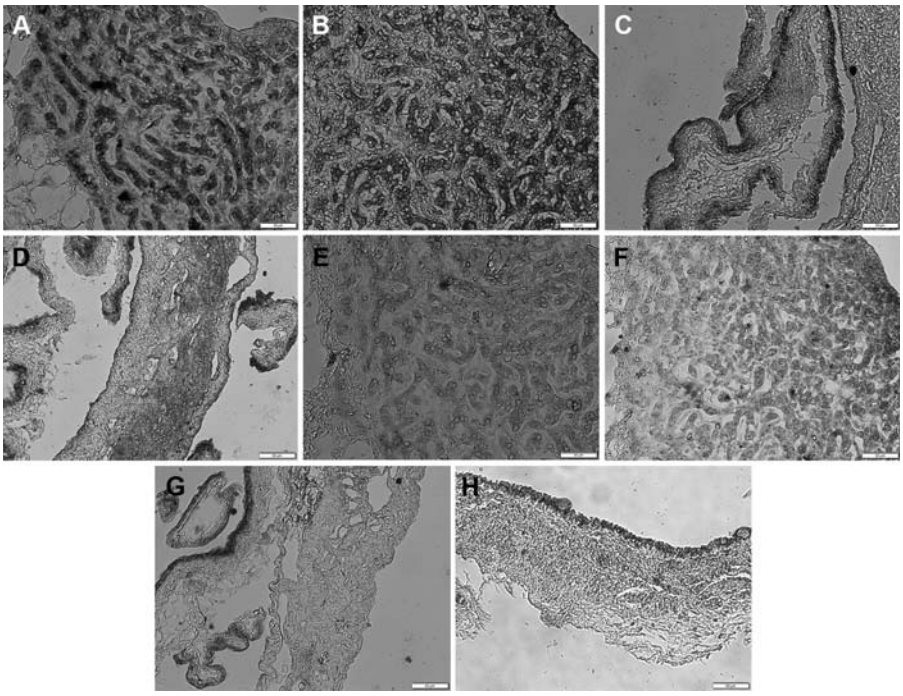

Fig. 5. Localization or nonlocalization of *Ardnd* and *vasa* expression in gonads of noninjected controls and 250-μM *dnd*-MO–injected juveniles at 210 days after fertilization determined by *in situ* hybridization. (A, B, E, F) Control noninjected group; (C, D, G, H) 250-μM *dnd*-MO–injected group. (A, C) Antisense *Ardnd* and (B, D) antisense *vasa* probes show positive signal in the control group and no signal in the 250-μM *dnd*-MO–injected group (germ cells are not present); (E, G) sense *Ardnd*, and (F, H) sense *vasa* probes (negative control) did not indicate any expression in both groups (scale bar = 50 μm). MO, morpholino oligonucleotide.

on serial sections of gonads of controls (Fig. 5A, B). No signal was observed in gonads of morphants with *Ardnd* and *vasa* antisense probes and all samples treated by *Ardnd* and *vasa* sense probes (Fig. 5C, D, E, F, G, H). These experiments confirmed the putative sterility of sterlet juveniles after *dnd*-MO treatment.

4. Discussion

The present study reported a functional method of sterlet sterilization using a knockdown agent, antisense MO. First, isolated fragments of *dnd* homologue gene in sterlet (*Ardnd*) were compared with the full-length sequence of Russian sturgeon (*Agdnd*), and the comparison showed the same sequence in the start codon region. Tissue-specific expression was detected by RT-PCR with positive *Ardnd* expression in gonads only. Second, *dnd*-MO was synthetized against a region spanning the ATG of *Agdnd* and injected into the vegetal pole of sterlet embryos for sterilization. Finally, the sterility and fertility of MO-injected fish and controls were analyzed by fluorescent microscopy and histology. Our data confirmed the sterility of the *dnd*-MO (250 μM)–injected group by loss of PGCs in the genital cavity. This is the first report on the successful sterilization among sturgeon species.

4.1. Importance of germ cells

In sexually reproducing organisms, PGCs are the only cells in developing embryos with potential to transmit genetic information to the next generation, undergo proliferation, and differentiate into functional gametes [13–16]. They migrate to genital ridge *via* chemotaxis; consequently, PGCs (or subsequent stages) are good candidates for induction of germ line chimerism through transplantation [2,17]. The production of germ line chimeras has the potential to generate offspring of endangered species by using common species that are adaptable to artificial conditions [14,18]. This germ line chimerism biotechnology could provide important advantages such as (1) shortening the reproduction period of sexually late-maturing species; (2) reducing the space for culture when small fish species used as hosts; (3) conserving germ cells for maintaining genetic resources; (4) retaining target species without keeping adult fish [18–20]. Our idea is to use sterlet, a common smaller sturgeon species with a shorter period of maturation and generation interval

(sexually mature in 5 years), as a host to produce gametes of a critically endangered species (donor) with a long reproductive cycle and large body size such as beluga (maturating in approximately 20 years). This could result in obtaining sperm and eggs of beluga four times faster than usual. Currently, this biotechnology is well developed for teleost (e.g., zebrafish, goldfish, or loach) by single PGC transplantation [2] or spermatogonia (SG) [21,22] and oogonia (OG) [23,24]. Before PGCs can be used for this purpose, their origin first must be determined and the character and migration pathway needs to be defined. This has been documented by Saito et al. [10], who found that PGCs in sturgeon are generated by the inheritance of germ plasm that is maternally deposited at the vegetal pole of the egg with holoblastic cleavage, which is similar to that in anurans. Then, PGCs migrate through the yolky cell mass toward the gonadal ridge, resembling the migration pattern of teleost. Surprisingly, PGCs of sturgeon transplanted into goldfish embryo at the blastula stage with completely different cleavage pattern (meroblastic) migrated to the genital ridge of the recipient [10]. Another possibility is the transplantation of subsequent stages of PGCs, the SG/OG. The advantage of SG/OG in comparison with PGCs is in their easy isolation from testes or ovary to obtain a large number of cells which can be directly microinjected into the body cavity of recipient fry to counteract the potential migration inability of transplanted germ cells [23,25]. To induce germ line chimera producing donor's gametes only, the host should be sterile. Okutsu et al. [21] transplanted SG into fertile rainbow trout and 50% of males produced donor-derived sperm but only 5.46 ± 3.34% offspring showed donor-derived progeny. In the case of females, 40% of them produced donor-derived eggs, from which only 2.14 ± 0.70% resulted in donor-derived progeny. Therefore, it is much more efficient to use sterile recipient which was the main objective of this study.

4.2. Problematic sterilization of sturgeon species

Besides the problematic reproduction of sturgeon, sterilization presents another complication for this popular topic in current research. Sturgeons differ in ploidy levels. This is probably the result of several genome duplications [26] and at least three independent polyploidization events during sturgeon evolution [6,27]. Also, allopolyploidization and autopolyploidization seem to be a recurring process in sturgeon with observed abnormal ploidy levels in these populations. Moreover, the possible fertility of odd ploidy levels in sturgeon is assumed [28]. Generally in fish, interspecific hybridization of distantly related species causes sterility by complications in the pairing of their chromosomes [29]. Hybrids between sturgeon species with same ploidy levels are considered to be fertile [6]. Moreover, the possible fertility of species with different ploidy levels was reported in several studies [6,30]. Another method of sterilization is by chemical treatment (e.g., busulfan) that inhibits gametogenesis at different developmental stages, but this does not guarantee 100% efficiency [22]. Altogether, polyploidization, hybridization, or chemical treatments are not functional or not proven methods of sturgeon sterilization. Nontransgenic genetic modification, a knockdown strategy to inhibit gene functions involved in early gonadal development, has been selected as practicable for sturgeon sterilization. The best gene candidate, expressed in germ plasm and responsible for the development and migration of PGCs, is *dnd* gene [8].

4.3. Identification of dnd gene

Dnd is a maternally inherited gene that is expressed in male and female gonads [8]. Therefore, unfertilized eggs were selected as suitable material for RNA extraction in this study. The *dnd* orthologs have already been described in invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* [8] and vertebrates such as chicken *Gallus gallus domesticus* [31], frog *Xenopus laevis* [8], mouse *Mus musculus* [32]. In fish, it has been initially identified in zebrafish *Danio rerio* [8] and subsequently in other teleosts such as medaka *Oryzias latipes* [33], loach *Misgurnus anguillicaudatus* [34], goldfish *Carassius auratus* [35], Atlantic salmon *Salmo salar* [36], Pacific bluefin tuna *Thunnus orientalis* [20], turbot *Scophthalmus maximus* [12], and the chondrosteans such as the Chinese sturgeon [9] and Russian sturgeon (Hagihara, unpublished data). The expression patterns of *Ardnd* were examined in several tissues of mature sterlet by RT-PCR in this study. The only signal was detected in gonads of both sexes, as was also reported in other fishes such as zebrafish [8], medaka [33], Atlantic salmon [36], Pacific bluefin tuna [20], turbot [12], and Chinese sturgeon [9]. This confirmed that *dnd* is a highly specific gene expressed in germ cells conserved in animals. All these studies suggested that *dnd* plays one of the main roles in germ cell development and is a useful germ cell marker for tracing their development. As a consequence, *dnd* was chosen as a probable candidate to design its knockdown agent, the *dnd*-MO, to block the migratory activity of PGCs.

Because full-length Russian *dnd* (*Agdnd*) was provided (Hagihara, unpublished data), sequenced fragment of *Ardnd* with the start codon region was substantial for MO design. The *Agdnd* and *Ardnd* sequences in the ATG region were similar which presents the possibility of inhibiting the *dnd* translation in two sturgeon species Russian (discussed in the following) and sterlet (present work). Multiple alignments showed that Russian *Agdnd* shared 97% identity with sequenced fragments of *Ardnd* and with full-length Chinese sturgeon *Asdnd* (KM 655832.1, [9]). By comparing the phylogenetic trees of *Agdnd* and *Asdnd*, we can classify these genes in a separate clade, as we have called "acipenseridae," from fish and tetrapods. This is probably due to the fact that acipenseridae belong to the oldest fish family, which appeared about 200 million years ago, and by all rights, they are called as living fossils in the literature [37,38].

4.4. Dnd-MO as a suitable tool for sturgeon sterilization

The gene knockdown was performed by an antisense MO as the best gene knockdown application used in studies of developing embryos [39]. Morpholino oligonucleotide is a synthetic chemical used to temporarily eliminate the function of genes by blocking pre–messenger RNA splicing

or messenger RNA translation, so that it generates nontransgenic genetically modified organisms [39,40]. We have designed an antisense MO against the *Ardnd* and *Agdnd* genes in the start codon region to induce elimination of PGCs before they enter the gonadal ridge. *Dnd*-MO was injected into sterlet embryos, the translation of *Ardnd* was inhibited, and it resulted in failure of PGCs to actively migrate. The development of somatic cells was not affected, as was reported for zebrafish [8].

Fluorescein isothiocyanate–dextran, injected into the vegetal pole of sturgeon embryos at one- to four-cell stage, where PGCs originate, was used for PGCs labeling according to Saito et al. [10]. Primordial germ cells appeared after reaching the tail-bud stage in controls. In morphants, a very low number of PGCs with stopped active migration were detected in yolk at 4 dpf (PGCs should stop their migratory activity in this stage) and none were found in later stages (21dpf), when PGCs of controls were already localized at germinal ridges. A similar finding was shown by Goto et al. [35]. In their study, the number of PGCs in *dnd*-MO–treated goldfish decreased at early somite stages and disappeared in the late somite stage. We concluded that treating the embryos with a 250-μM concentration of *dnd*-MO was sufficient to induce complete loss of sterlet PGCs in this study. This dose range is a little higher than that used in other fish species (50–200 μM, [35]), probably due to larger size of sturgeon eggs in comparison with teleost ones. Moreover, we tested the function of *dnd*-MO in Russian sturgeon, and it affected the development of PGCs and survival rate of Russian sturgeon in the same way as described in sterlet (data not shown).

To improve the strength of the experiment, the loss of germ cells in morphants was confirmed by histologic analysis. The gonadal development of controls at 60, 150, and 210 dpf (noninjected *dnd*-MO fish) did not reveal significant differences in the gonad morphology or the rate of their development in comparison with other sturgeons [1,41–43]. Finally, the expression of *Ardnd* in gonads of 210-dpf-old morphants and controls was examined by RT-PCR and ISH. The positive expression was detected only in the control group.

In conclusion, we sterilized sterlet by *dnd*-MO. The successful ablation of PGCs was confirmed by counting of FITC-labeled PGCs under fluorescent microscopy, by histologic examinations, and gene expression analyses. Our results provided a very useful tool of sterlet sterilization for future experiments of surrogate reproduction *via* germ line chimerism, where a sterile host is required. This could be a powerful method for reproduction of these endangered species.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2015.07.003.

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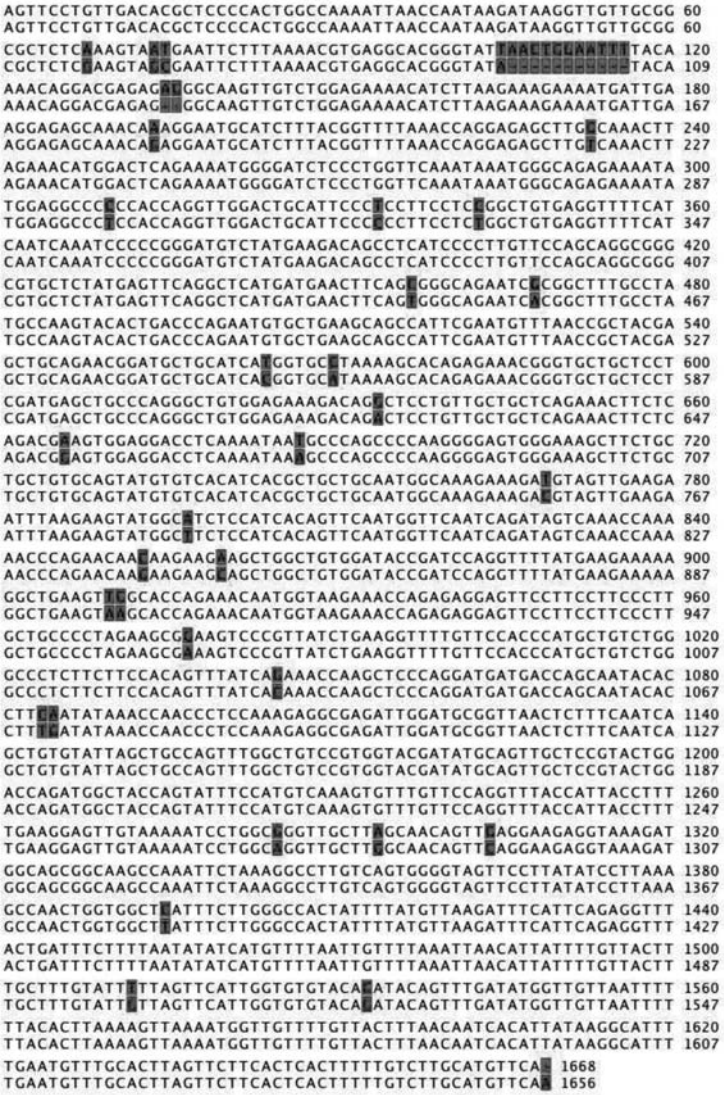

Supplementary Fig. 1. Alignment results of *Agdnd* (first sequence) with fragments of *Ardnd* (second sequence). Red rectangles indicate missing or different parts in *dnd* seqeunces.

CHAPTER 7

GENERAL DISCUSSION

Induction of germ-line chimerism is a powerful method to enhance the reproduction of endangered species, species commercially valuable or species with problematic reproduction using common species easily adapted to artificial reproduction as the surrogate host (Okutsu et al., 2006b; Yamaha et al., 2007). To be able to establish this biotechnology, several studies focused on documentation of donor and host embryonic and larval development, characterization of GC development and recording their migratory activity, sterilization of the host, isolation and cryopreservation of donor germ cells, have been performed. All these crucial points were the main objective of present study.

The whole study provided the focus on two different fish species. First, our commercially valuable fish from infraclass Teleostei, the tench, where we would like to apply the present studies and our current knowledge to create a germ-line chimera within cyprinids by transplantation of tench (donor) GC to smaller and faster-reproducing fish species as white cloud mountain minnow. Secondly we focused on the endangered species of large body size with long reproductive cycle, the sturgeons. In this case, we have chosen sterlet as a host, providing an advantage of shorter generation interval and smaller body size, to produce gametes of donor, a critically endangered species of large body size with long reproductive cycle, such as beluga. This innovative technology could result in obtaining sperm and eggs in shorter time from small-bodied host.

In the first study we provided important information on morphology and ultrastructure of beluga (as future donor of GC) spermatozoa structure by scanning and transmission electron microscopy to increase knowledge of evolutionary and taxonomic relationships among sturgeons (Linhartova et al., 2013). Our results have confirmed the suggestion that the morphology and ultrastructure of sturgeon spermatozoa is of taxonomic value (Jamieson, 1991; DiLauro et al., 2001; Psenicka et al., 2007) and can be a basis for defining phylogenic relationships among these species. Moreover, the similarity in spermatozoa morphology, along with the close genetic relationship, supports the suggestion of findings of many researchers (Birstein and DeSalle, 1998; Zhang et al., 2000; Ludwig et al., 2001) that beluga is closely related to genera *Acipenser* and might not belong in genera *Huso*, where is presently assigned.

The preliminary but necessary experiment for future transplantation of germ cells, mostly the PGCs, is the study of their origin and migration activity. In the second study we described embryonic and larval development of the tench with detailed characterization of each developmental stage (Linhartova et al., 2014a) in comparison with previously reported data on tench development by Penaz et al. (1981) or Korzelecka-Orkisz et al. (2009), to describe the germ cell migration patterns. Our result demonstrated that tench development follows the general flow of events characteristic for teleosts (meroblastic cleavage) such as zebrafish (Kimmel et al., 1995), ice goby (Arakawa et al., 1999), goldfish (Yamaha et al., 1999), and loach (Fujimoto et al., 2006) and developmental stages were determined according to Kimmel et al. (1995). PGCs were identified by injecting synthesized mRNA, combining green fluorescent protein and the zebrafish *nos 1* 3′UTR (a germ-line specific marker), under the blastodisc of embryos at the 1-4-cell stage. The migration pathways of fluorescent PGCs were detected from 100% epiboly, compared to other fish species (zebrafish, pearl danio, loach, Japanese eel), in which PGCs were observable at around 50% epiboly and in ice goby at 90% epiboly (Saito et al., 2006, 2011). PGCs formation was comparable to that observed in medaka, goldfish, and ice goby during the segmentation period (Saito et al., 2006). Tench PGCs were observed to the end of the hatching period (184 hpf) in 69.3% of injected embryos, where migrated to the site of future gonads. These results provide evidence that routes of PGC migration are species-specific.

In a similar way, origin and migration routes of sturgeon PGCs were successfully documented by Saito et al. (2014). They revealed an injection technique for sturgeon eggs which allowed visualization of germ plasm with PGCs. They demonstrated that the PGCs occur at the vegetal pole of the egg (on opposite pole in comparison with teleosts) and they migrate on the yolky cell mass toward the animal pole and genital ridge of developing embryo. They suggest that PGCs characteristic in sturgeon is similar to that of anurans, but the migration pattern resembles that of teleosts (Saito et al., 2014).

Not only PGCs (precursors of germ cells) have the potential to transmit genetic information to the next generation (Kawakami et al., 2010), but also their subsequent stages, the SG/OG, as first germ cells in testes/ovary (Linhartova et al., 2014b, Psenicka et al. 2015). All these cells have a great potential for utilization in cryopreservation and gene banking (Okutsu et al., 2006b; Yamaha et al., 2007). The disadvantage of SG/OG use for transplantation is their partial loss of migration activity (Okutsu et al. 2006a, b; Yoshizaki et al., 2010), therefore they have to be microinjected directly to area of genital ridge of body cavity of recipient to avoid the potential migration inability of transplanted GC (Yoshizaki et al., 2010; Psenicka et al., 2015). On the other hand, their big advantage when compared with PGCs is their easy isolation from testes/ovary resulting in yield of a large number of GC. Moreover, these early stages of germ cells are small enough to be well-suited to cryopreservation. Together with their high level of plasticity it makes their preservation a promising tool for maintaining genetic resources. Therefore practical methods of their isolation and also cryopreservation were established in both fish species examined in this study. GC were dissociated with enzymes – collagenase/trypsin (Bellve et al., 1977; Lacerda et al., 2006; Okutsu et al., 2006b), 0.1% collagenase and trypsin mixture was used in tench (Linhartova et al., 2014b) whereas 0.3% trypsin was used in Siberian sturgeon (Psenicka et al., 2015). Testicular/ovarian cells were separated by Percoll gradient, as a simple method that does not require complex laboratory equipment, according to Lacerda et al. (2006) and Psenicka et al. (2012). The highest proportion of tench GC (62.2%) was obtained from the 30% layer of Percoll (Linhartova et al., 2014b) and isolated GC of Siberian sturgeon (79.4% and 70.8% testicular and ovarian cells) from 10–30% layer (Psenicka et al., 2015).

Significant inter-species variations as well as significant within-species differences in cryoprotectant effectiveness have been reported in fish spermatozoa (Alavi et al., 2012). Therefore the effectiveness of the extender and cryoprotectant must be optimized for each fish species. Early stages of germ cells were cryopreserved in an extender composed of PBS with 0.5% BSA, 50 mM d-glucose, and containing 1.5 M cryoprotectant in the pre-programmed PLANER Kryo10 series III using a cooling protocol from + 10 °C to – 80 °C at a rate of 1 °C.min^{-1}. The effect of different cryoprotectants – methanol, dimethyl sulfoxide, dimethyl sulfoxide + propanediol (1:1), glycerol, ethylene glycol, and dimethylacetamid was assessed, and the results were evaluated by comparing the percentage of viable frozen/thawed GC by ANOVA, Tukey's HSD test ($P < 0.05$) (Linhartova et al., 2014b). In the case of tench, almost the same viability rates were obtained with no significant differences among tested cryoprotectants, indicating high stability of GC in cryoprotectants. Nevertheless, GLY at a concentration of 1.5 M was associated with the highest survival rate 57.69% of thawed tench GC (Linharttova et al., 2014b). In the case of Siberian sturgeon, 1.5 M EG was evaluated as the most effective with survival rate of GC 61.75% (Psenicka et al., 2012). Taken together, effectiveness of the extender and cryoprotectant is species-specific as well as individually dependent as was similarly observed in fish spermatozoa (Alavi et al., 2012). We can conclude that present results represent a feasible method for GC isolation and cryopreservation usable in hatchery practice as well as in research. Furthermore, these data are also considered preliminary results for application in fish bioengineering by transplanting cryopreserved GC into a sterile xenogeneic recipient.

Finally, we focused on sterilization of fish, which is required for surrogate reproduction via germ line chimerism. In fish, sterilization is usually achieved by triploidization (Vandeputte et al., 2009), hybridization (Piferrer et al., 2009; Havelka et al., 2013) or by chemical treatment in form of baths or diets (Lacerda et al., 2006). Tench could be sterilized by triploidization (Flajshans et al., 2010). On the other hand, none of mentioned method does quarantee 100% efficiency in sturgeons. Beside their problematic reproduction, the effective sterilization or sterility/fertility of hybrid specimens is another stumbling-block and a hot topic in current research. Sturgeons differ in ploidy levels (e.g. sterlet is a functional tetraploid) and number of chromosomes (Peng et al., 2007; Havelka et al., 2011, 2013, 2014). Hybrids of sturgeon species with same ploidy levels, as well hybrids of species of different ploidy level (Flajshans and Vajcova, 2000) are considered to be fertile (Havelka, 2013). The non-transgenic knockdown strategy to inhibit the function of genes by using a KD agent the morpholino antisense oligonucleotide (Draper et al., 2001; Summerton, 2007) was applied. *Dead end* gene (*dnd*), a vertebrate-specific gene encoding an RNA-binding protein crucial for formation, migration and survival of PGCs (Weidinger et al., 2003) was chosen as a target gene for KD. For this purpose, a *dnd* homologue of Russian sturgeon (*Agdnd*), having identical sequence in start codon region with isolated fragments of sterlet *dnd* (*Ardnd*) was applied (Linhartova et al., 2015, Chapter 6). *Dnd*-MO together with Fluorescein isothiocyanate (FITC) conjugated with dextran, commonly used tracer dye for labeling cells (Saito et al., 2014), were injected into the vegetal region of 1-4-cell stage sterlet embryos. FITC was injected to validate the injection method and labeling of PGCs in control groups. The successful ablation of PGCs was confirmed by histology and fluorescent microscopy in sterlet embryos, larvae and juveniles (Linhartova et al., 2015, Chapter 6). Our results reported a method for successful sterilization in sturgeon species and provided a cornerstone of very powerful tool for reproduction of these endangered species via germ line chimerism and surrogate production.

Finally, this thesis presents several studies with differing focus of research but with one target goal – to induce germ-line chimerism in fish. All these results are prerequisite of future application and development of surrogate production in these species.

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ENGLISH SUMMARY

Micromanipulation and cryopreservation of germ cells in fish

Zuzana Linhartová

The induction of germ-line chimerism is an expanding focus of fisheries research. This technique has potential to enhance the production of gametes of species that are commercially valuable, endangered, species with problematic reproduction, using a more common or easily available species or species adapted to artificial reproduction as a surrogate host. The main goal of this technology is to establish a small-bodied surrogate broodstock producing functional donor gametes based on germ cell transplantation. Extending preliminary experiments, including documentation of donor/host embryonic and larval development, characterization of germ cells enriched by documentation of their migratory activities, sterilization of the host, isolation and cryopreservation of donor germ cells, are key factors for launching this biotechnology. All these crucial points were the main objective of the present work.

The whole thesis provides a focus on two different fish species. First, our commercially valuable fish, the tench, where we would like to apply our current knowledge to create a germ-line chimera within cyprinids by transplantation of tench germ cells to smaller and faster-reproducing fish species as white cloud mountain minnow. Second we focused on the endangered species (listed in IUCN Red List) of large body size with long reproductive cycle, the sturgeons. In this case, we have chosen sterlet as a host, providing an advantage of shorter generation interval and smaller body size, to produce gametes of donor, a critically endangered species of large body size with long reproductive cycle, such as beluga. This innovative technology could result in collection of sperm and eggs in shorter time from small-bodied host.

In tench we first focused on embryonic and larval development documentation together with description of origin and migration pathways of primordial germ cells (PGCs). PGCs represent a powerful tool for creation a germ-line chimera within fish species because they transmit genetic information to the next generation (Linhartova et al., 2014a). Secondly, we reported a practical technique for isolation and cryopreservation of early stages of germ cells (GC), including spermatogonia (SG) and spermatocytes (Linhartova et al., 2014b).

In case of sturgeons, Saito et al. (2014) firstly described the origin and migration patterns of sturgeon PGCs deposited at the vegetal pole of the egg similar to that in anurans. Secondly, Psenicka et al. (2015) reported isolation and cryopreservation of female and male GC, SG from testes and OG and pre-vitellogenic oocytes from ovary, of 2–4-year old Siberian sturgeon. Moreover the isolated GC were transplanted into host (sterlet) and process of transplantation resulted in successful colonization of sterlet genital ridge. The potential host for germ-cell tranplantation, sterlet, was sterilized by knock-down of germ cell specific gene, the *dead end* gene, by the morpholino antisense oligonucleotide (MO) agent (*dnd*-MO). These results reported the first known and functional method of sturgeon sterilization (Linhartova et al., 2015). We provided important information on morphology and ultrastructure of beluga spermatozoa structure by scanning and transmission electron microscopy to increase knowledge of evolutionary and taxonomic relationships among sturgeons (Linhartova et al., 2013).

Finally, this thesis presents several studies with differing focus of research but with one target goal – to induce germ-line chimerism in fish. All these results are prerequisite of future application and development of surrogate production in these species.

CZECH SUMMARY

Mikromanipulace a kryoprezervace zárodečných buněk ryb

Zuzana Linhartová

Vytváření chimér zárodečných linií je v současné době jedním z poutavých témat výzkumu v oblasti rybářství. Tato metoda má potenciál zabezpečit nebo zvýšit produkci gamet druhů, které jsou komerčně velmi ceněné, ohrožené nebo druhů s problematickou reprodukcí, a to za použití běžně dostupného druhu přizpůsobeného k umělému výtěru jako producenta těchto gamet.

Hlavním cílem této technologie je získání generačních ryb malých rozměrů (host) produkující reprodukčně funkční gamety druhu původního (donora) po transplantaci zárodečných kmenových buněk donora do hosta. Klíčovými faktory pro vytvoření této biotechnologie je velké množství předběžných experimentů: dokumentace embryonálního a larválního vývoje donora i hosta, charakterizace zárodečných kmenových buněk společně s dokumentací jejich migračních aktivit, sterilizace hosta, izolace a kryoprezervace zárodečných buněk donora. Všechny tyto pokusy byly hlavním cílem této práce.

V této práci jsme se zabývali dvěma odlišnými taxonomickými skupinami ryb. Nejprve naší hospodářsky využívanou rybou, línem obecným, kde bychom chtěli uplatnit své dosažené znalosti k vytvoření chiméry zárodečné linie u kaprovitých ryb za pomoci transplantace zárodečných buněk lína (donora) do menšího a rychleji se rozmnožujícího druhu, kterým je kardinálka čínská. Dále jsme se zaměřili na jesetery, skupinu ryb zahrnující celou řadu ohrožených druhů (evidovaných v Červené knize ohrožených druhů (IUCN)), ryby dosahující velkých rozměrů a zpravidla s dlouhým reprodukčním cyklem. V tomto případě jsme si vybrali jesetera malého jako hosta kvůli kratšímu reprodukčnímu intervalu a menší velikosti těla, k produkci gamet donora, kriticky ohroženého druhu s dlouhým reprodukčním cyklem, jakým je vyza velká. Touto inovativní technologií bychom mohli získat spermie a jikry v kratším časovém intervalu a od menšího druhu.

V případě lína obecného jsme se nejprve zaměřili na dokumentaci jeho embryonálního a larválního vývoje společně s popisem původu a migračních aktivit primordiálních zárodečných buněk (PGCs). Tyto buňky byly vybrány jako vhodný prostředek pro vytvoření chiméry zárodečné linie v rámci druhu, jelikož nesou genetickou informaci obou rodičů a jsou schopné samovolně migrovat do míst výskytu budoucích gonád (Linhartova a kol., 2014a). Poté jsme vyvinuli praktické metody pro izolaci a kryopreservaci pozdějších vývojových stádií kmenových zárodečných buněk (GC), spermatogonií (SG) a spermatocytů (Linhartova a kol., 2014b).

V případě jeseterovitých ryb Saito a kol. (2014) popsali migrační aktivitu i samotný původ PGCs, který se soustředuje do vegetativního pólu jikry, stejně jako je to známo u obojživelníků. Dále Pšenička a kol. (2015) popsali izolaci a kryopreservaci GC, zastoupených převážně SG a OG a previtellogeními oocyty, 2–4letých jeseterů sibiřských. Izolované GC byly transplantovány do hosta (jesetera malého) a úspěšně kolonizovaly jeho zárodečnou rýhu. Na závěr jsme se zabývali sterilizací hosta, jesetera malého, potenciálního hosta pro transplantaci zárodečných buněk donora. Snahou bylo dosáhnout sterility pomocí knockdownu specifického genu zárodečných buněk látkou – *morpholino antisense oligonukleotid* (MO) – *dnd*-MO. Naše výsledky popisují první spolehlivě fungující způsob sterilizace jeseterovitých ryb (Linhartova a kol., 2015). Navíc jsme publikovali důležité informace o morfologii a ultrastruktuře spermií vyzy velké pomocí skenovací a transmisní elektronové mikroskopie, rozšiřující znalosti o stavbě spermií a týkající se i evolučních a taxonomických vztahů mezi jesetery (Linhartova a kol., 2013).

Tato práce představuje několik studií s odlišnými zaměřeními, ale s jedním hlavním cílem – vytvoření chimér zárodečných linií u ryb. Všechny tyto výsledky jsou předpokladem pro budoucí aplikaci této technologie.

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- COST – LD14119 – Aquatic gametes as model for fundamental cell motility studies (responsible leader Martin Pšenička, Ph.D.)
- GAJU 114/2013/Z – New methods and biotechnological approaches in fish reproduction and genetics (responsible leader Prof. Otomar Linhart)
- GAJU 080/2013/Z – Induction of germ-line chimerism by transplantation of tench (*Tinca tinca*) primordial germ cells into white cloud mountain minnow (Tanichthys albonubes) (responsible leader M.Sc. Zuzana Linhartová)
- P502/13/26952S – Induction of chimerism by transplantation of germ stem cells in critically endangered sturgeons as a tool of their conservation (responsible leader Martin Pšenička, Ph.D.)

LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Linhartova, Z., Saito, T., Kaspar, V., Rodina, M. Hagihara, S., Psenicka M., 2015. Sterilization of sterlet *Acipenser ruthenus* by using knock down agent, antisense morpholino oligonucleotide, against *dead end* gene. Theriogenology doi: 10.1016/j.theriogenology.2015.07.003.

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Applied methodologies, patents, pilot plants, verified technologies

Psenicka, M., Saito, T., Rodina, M., **Linhartova, Z.**, Flajshans, M., 2012. Isolation and cryopreservation of sturgeon spermatogonia and oogonia in order to preserve genetic resources. Edition of Methods (Technological series), FFPW USB, Vodňany, Czech Republic. No. 129, 22 pp. (in Czech).

Abstracts and conference proceedings

Linhartova Z., Rodina, M., Guralp, H., Gazo, I., Saito, T., Psenicka, M., 2014. Detailed documentation of embryogenesis of tench (*Tinca tinca*) enriched by visualization, migration, isolation and cryopreservation of early stages of germ cells. In: Aquaculture Europe 2014, Abstract Book, Donostia San Sebastian, Spain, p. 22.

Linhartova, Z., Kaspar V., Saito, T., Fujimoto T., Arai K., Yamaha E., Psenicka M., 2014. Induction of germ-line chimerism within sturgeons. In: International Conference World Aquaculture, Abstract Book, Adelaide, Australia, p. 80.

Linhartova, Z., Rodina, M., Guralp, H., Saito, T., Ozturk, D., Psenicka, M., 2013. Isolation and cryopreservation of spermatogonia of tench (*Tinca tinca*). In: Diversification in Inland Finfish Aquaculture (DIFA II), Abstract Book, USB FFPW Vodňany, Czech Republic, p. 86.

Linhartova, Z., Rodina, M., Nebesarova, J., Cosson, J., Psenicka, M., 2013. Morphology and ultrastructure of beluga (*Huso huso*) spermatozoa and a comparison with related sturgeon. In: The 4th International Workshop on the Biology of Fish Gametes (Fishgametes 2013), Abstract Book, Algavre, Portugal, p. 64.

Linhartova, Z., Psenicka, M., Kaspar, V., Saito, T., Fujimoto, T., Arai, K., Yamaha, E., 2012. Micromanipulations with sturgeon germ cells. In: World Aquaculture Society Conference AQUA 2012, Abstract Book, Prague Congress Centre, Prague, Czech Republic, p. 80.

Linhartova, Z., Saito T., Psenicka, M., 2012. Embryogenesis, visualization and migration of primordial germ cells in tench (*Tinca tinca*). In: VIth Workshop on Biology and Culture of the tench, Abstract Book, Pisek, Czech Republic, p. 22.

Linhartova, Z., Saito T., Psenicka, M., 2012. Embryogenesis, visualization and migration of primordial germ cells in tench (*Tinca tinca*). In: Domestication in Finfish Aquaculture, Abstract Book, Olsztyn, Poland, p. 81.

Psenicka, M., **Linhartova, Z.**, Kaspar, V., Saito, T., 2012. Induction of chimerism by transplantation of germ stem cells in sturgeons. In: XIII Czech Ichthyologic Conference, Abstract Book. Hotel Vltava, Cervena nad Vltavou, Czech Republic, p. 22.

TRAINING AND SUPERVISION PLAN DURING STUDY

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Applied hydrobiology	2012
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World Aquaculture Society Conference AQUA 2012, 1–5 September 2012, Prague Congress Centre, Prague, Czech Republic. (oral presentation)	2012
Domestication in Finfish Aquaculture, 23–25 October 2012, Olsztyn, Poland. (poster presentation)	2012
The 4th International Workshop on the Biology of Fish Gametes (Fishgametes 2013), 17–20 September 2013, Algavre, Portugal. (poster presentation)	2013
Diversification in Inland Finfish Aquaculture (DIFA II), USB, FFPW, 24–26 September, 2013, Vodnany, Czech Republic. (poster presentation)	2013
International Conference World Aquaculture, 7–11 June 2014, Adelaide, Australia. (oral presentation)	2014
Aquaculture Europe 2014, 14–17 October 2014, Donostia San Sebastian, Spain. (oral presentation)	2014

Foreign stays during Ph.D. study	**Year**
Laboratory of Fish Physiology and Genomics, National Institute of Agronomical Research (INRA), Rennes, France (under supervision of Dr. Florence Le Gac and Dr. Catherine Labbe)	2013
Laboratory of Aquaculture Genetics & Genomics, Faculty of Fisheries Sciences, Hokkaido University (under supervision of Prof. Katsutoshi Arai and Prof. Takafumi Fujimoto)	2013

Teaching	**Year**
Biotechnologies – lecture, supervision of experimental part, guarantors of subject: Martin Pšenička, Ph.D., and Vojtěch Kašpar, Ph.D.	2013 and 2014
Chemistry I, lecture and leading of laboratory work and exercices, guarantor: Kateřina Grabicová, Ph.D.	2013

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B.Sc. Dvořák Matěj	Identification and isolation of primordial gonocytes in sturgeon	2014
Petr Dobrovolný	Rescue of critically endangered fish species by manipulating with spermatogonia and oogonia	2015

B.Sc. students supervision		**Year**
Jaroslav Hrdlička	Verifying of the fertility of hybrids sturgeon using histology of gonads	2015

Summer school students supervision		**Year**
Nela Nováková	Ultrastructure of sperm in beluga (*Huso huso*)	2012
Bogdan Altukhov	Ultrastructure of sperm in beluga *(Huso huso*)	2012
M.Sc. Mamen Vilchez Olivencia	Isolation and cryopreservation of fish spermatogonia	2013
Aybike Demirtas	Isolation and cryopreservation of fish spermatogonia	2013
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2011 Course of electron microscopy, Biology Center, The Academy of Sciences of the Czech Republic, Ceske Budejovice

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