University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
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DIPLOMA THESIS

Isolation of early stages of germ cells in pikeperch (*Sander lucioperca*)

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Rules for elaboration:

Germ stem cells, such as priomordial germ cells or subsequently early stages of oogonia or spermatogonia, are precursors of gametes, which keep the ability to differentiate into both types of gametes, and undergo proliferation and recombination of genetic information during oogenesis or spermatogenesis, respectively. Moreover the cells have a potential for isolation, cryopreservation and inter or intra specific transplantations. Therefore they are value for gene banking and surrounding production of gametes via germ-line chimera.
This study performs the first crucial step for generation of germ-line chimera, which is isolation of spermatogonia from a fresh water fish species, pikeperch. In further experiments, spermatogonia of pikeperch can be used for transplantation into a close related marine species such as meagre (both are representatives of *Perciformes* order).

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List of specialized literature:

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1. Introduction

Pikeperch (*Sander lucioperca*) is a valuable recreational and commercial fish species in Europe, which is generally produced in ponds, in polyculture with common carp or in recirculation systems (RAS) using commercial feed (Lappalainen et al., 2003, Molnár et al., 2004, Philipsen and Van der Kraak, 2008). The supply of pikeperch is increased significantly last decade (FAO, 2012). On the other hand stable fry production depends on appropriate determination of maturation which needs sensitive care and expertise (Rónyai, 2007).

Cryopreservation of fish embryos might be a useful tool in aquaculture and in the management of wild stocks for fish farming. But cryopreservation of fish eggs and embryos is accepted to be a crucial problem in cryobiology. The eggs of most species are complex when compared to sperm cells and they also have very low membrane permeability (Zhang et al., 1993).

On the other hand primordial germ cells (PGCs) and early stages of oogonia (OG) or spermatogonia (SG) are precursors of gametes and they able to continue gametogenesis (spermatogenesis or oogenesis) after transplantation (T Okutsu et al., 2006). Their potential to differentiate into functional gametes, and transmit genetic information to the next generation makes them suitable for cryopreservation and surrogate reproduction studies by germline chimera (Yoshizaki et al., 2003). In theory, spermatogonia can be preserved in two ways, cryopreservation or long-term culture (Woelders and Rooij, 2002).

Spermatogonial stem cells (SSCs) are formed from the primordial germ cells (PGCs). SSCs in fish regenerate and differentiate themselves to mature spermatids trough spermatogenesis, which occur in a cyst in the seminiferous tubules contrary to mammals (Fig. 1) (Schulz et al., 2005). The formation of the cysts is due to the encapsulation of sole primary spermatogonium, eventually transforming in to isogenic germ cell clone through concurrently divisions, by a sertoli cell(s) (Schulz et al., 2005, Vilela et al., 2004).

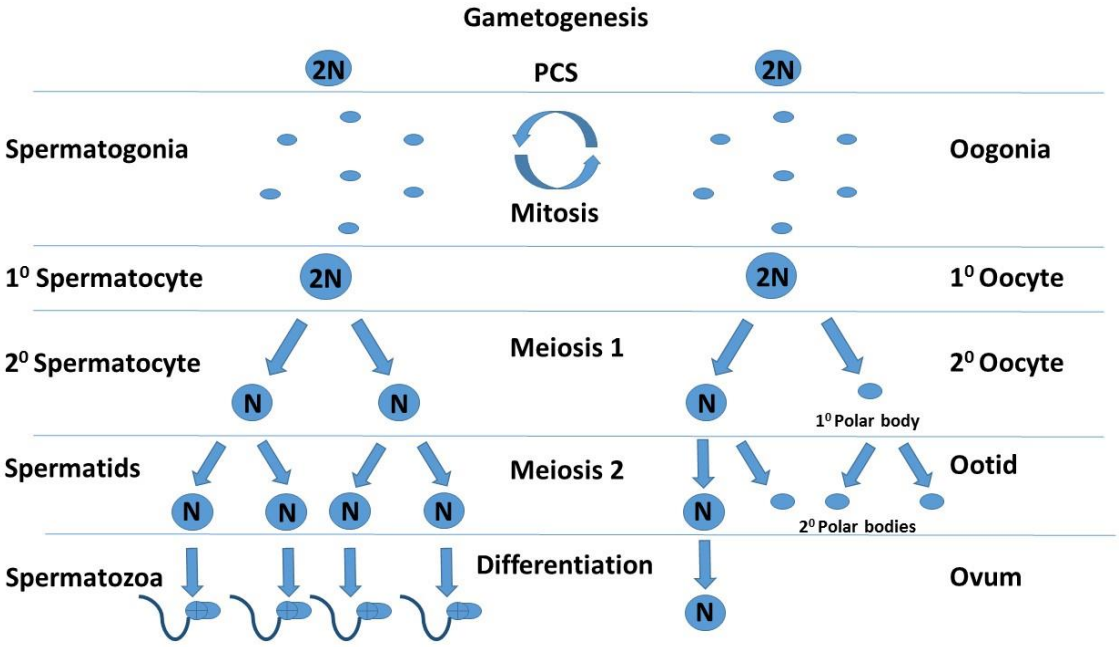


Fig.1 Original figure shows animal gametogenesis in different stages. Shape and size in each stage may not be accurately represented.

Brinster (2002) developed an advanced technique for spermatogonial transplantation, used for studying male reproductive biology, especially parts related to spermatogenesis and the stem cell biology. The technique covers the spermatogonial stem cells isolation from a donor animal and the transplantation of these cells into a recipient. These cells will develop and form mature spermatozoa with the genetic characteristic of donor (Brinster, 2002). Particular importance of this technique is, its promising implementations in biotechnology, transgenic animals, and the preservation of the genetic stock of valuable animals or endangered species (Lacerda et al., 2006). Recently many other researchers have studied on this field by using different techniques. Their methods and success ratios has been summarized on Table 1.

Table 1. Shows recent studies about chimera production whit host and donor species, host sterilization method, isolation method, germ cell identification method, chimerism identification method and their success rates.

Host Sp.	Donor Sp.	Host sterilization	Isolation of germ cells	Identification of Germ cell	Identification of chimerism	Success	Reference
Albino zebrafish (Danio rerio)	Wild Type zebrafish (Danio rerio)	no sterilization	blastomere transplantaton (no isolation)	fluorescein dextran	pigmentation + progeny genotyping	5/28 chimeric fish produced donor-derived progeny at frequencies of 1-40%	(Lin et al., 1992)
Crucian carp (Carassius auratus langsdorfii)	Diploid Goldfish (Carassius auratus)	no sterilization	blastomere transplantaton (no isolation)	blastomere transplantaton (no identification)	FITC-Dextran-biotin + Progeny	chimeric fish produced donor-derived progeny at frequencies of 3.4 to 93.2%	(Yamaha et al., 2001)
Wild type rainbow trout (Oncorhynchus mykiss)	Dominant orange-colored Rainbow trout (Oncorhynchus mykiss)	no sterilization	blastomere transplantaton (no isolation)	FITC-dextran	FITC, Progeny, pigmantation	6/19 chimeras produced donor delived progeny in range from 0.3 to 14%.	.(Takeuchi et al., 2001)
Orange Type loach (Misgurnus anguillicaudatus)	Wild type loach (Misgurnus anguillicaudatus)	no sterilization	blastomere transplantaton (no isolation)	no identification	melanophore, pigmentation of offspring	1/4 chimeras produce donor-derived gametes	(Nakagawa et al., 2002)
Rainbow trout (Oncorhynchus mykiss)	Rainbow trout (Oncorhynchus mykiss) orange-colored	no sterilization	manually	vasa-GFP	vasa-GFP, PCR for GFP, pigmentation	17/79 fish contained donor PGC 30 dpt; 2/12 males produce donor-derived sperm.	(Yoshizaki et al., 2003)
Rainbow trout (Oncorhynchus mykiss)	transgenic rainbow trout (Oncorhynchus mykiss)	no sterilization	no isolation	vasa-GFP	vasa-Gfp	13/26 males produced 5.46 ± 3.34% donor-derived progeny 16/40 females produced 2.14 ± 0.70% donor-derived progeny	(Tomoyuki Okutsu et al., 2006)
Masu salmon (Oncorhynchus masou)	Transgenic Rainbow trout (Oncorhynchus mykiss)	triploidization	no isolation	vasa-GFP	vasa-Gfp, genotyping	10/29 males produce donor sperm and 8/50 females vitellogenic oocytes and 5/50 females ovulated donor eggs	(Okutsu et al., 2008)
Zebrafish (Danio rerio)	Pearl danio (Danio albolineatus)	Antisense dead end morpholino oligonucleotide	Manually	(GFP)-nos1 30UTR mRNA	vasa, genotyping, RT PCR	50% PGC migrated into genital ridge and produce donor-derived gametes	(Saito et al., 2008)
Zebrafish (Danio rerio)	Zebrafish (Danio rerio)	no sterilization	Manually	(GFP)-nos1-3'UTR strand-capped mRNA	(GFP)	32% of cultured PGCs detected 2 dpf	(Higaki et al., 2010)

Host Sp.	Donor Sp.	Host sterilization	Isolation of germ cells	Identification of Germ cell	Identification of chimerism	Success	Reference
Nile Tilapia (Oreochromis niloticus) - Chitralada tilapia strain	Nile Tilapia (Oreochromis niloticus) - red tilapia strain	Busulfan	Percoll gradient	PKH26	Different strain, PKH26, genotyping	34/38 of PKH26 labelled cells in different stages, 2/32 males producing sperm	(Lacerda et al., 2010)
Zebrafish, Pearl danio, Goldfish, Loach	Zebrafish (Danio rerio)	no sterilization	manually	GFP-nos1 3'UTR mRNA	GFP-nos1 3'UTR mRNA	99/343, 84/209, 167/263, 44/102 PGCs in gonadal region 2 dpf	(Saito et al., 2010)
Nibe croaker (Nibea mitsukurii)	Yellowtail (Seriola quinqueradiata)	no sterilization	no isolation	PKH26 + vasa	PKH26 + genotyping (vasa) + (anti-PCNA)	72/88 spermatogonia incorporated and proliferated 3 wpt. 0/10 females and 1/10 males after 11 mopt, 0/92 male produced donor derived gametes	(Higuchi et al., 2011)
Zebrafish and danio hybrid	Zebrafish (Danio rerio)	Hybridization	Percoll gradient	vasa-DsRed2; EGFP-bactin	vasa-DsRed2; EGFP-bactin	12/67 males produce donor sperm	(Wong et al., 2011)
Zebrafish (Danio rerio)	Japanese eel (Anguilla japonica)	no sterilization	manually	GFP-nos1 3UTR mRNA	GFP-nos1 3UTR mRNA	32/75 PGC localized at gonadal region 2 dpf	(Saito et al., 2011)
Yellowtail (Seriola quinqueradiata)	Yellowtail (Seriola quinqueradiata)	no sterilization	no isolation	PKH26 fluorescent dye	PKH26 + ISH (vasa)	66.7% incrporated donor germ cells at 28 dpt. 590 injected, 30 get mature, 2 males produced donor sperm	(Morita et al., 2012)
Nile tilapia (Oreochromis niloticus)	Nile tilapia (Oreochromis niloticus)	Busulfan	cell culture	Immunostaining	PKH26 - Gfra1	100% incorporated	(Santos Nassif Lacerda et al., 2013)
Chub mack erel (Scomber japonicas)	Pacificbluefin tuna (Thunnus orientalis)	no sterilization	no isolation	dead end gene	PKH26	3/56 fish incorporated testicular cells 21 dpt	(Yazawa et al., 2013)
Goldfish (Carassius auratus auratus)	Sterlet (Acipenser ruthenus)	no sterilization	manually	GFP-nos1 3UTR mRNA	GFP-nos1 3UTR mRNA	2/36 PGCs localized in gonadal region 3 dpf	(Saito et al., 2014)
Nile Tilapia (Oreochromis niloticus)	Nile Tilapia (Oreochromis niloticus)	no sterilization	no isolation	medaka β-actin/enhanced green fluorescent protein (EGFP)	PKH26, DAPI, genotyping	2/5 males produce milt of donor, 1/3 oocytes of donor.	(Farlora et al., 2014)
Sterlet (Acipenser ruthenus)	Siberian sturgeon (Acipenser baerii)	no sterilization	Percoll gradient	PKH26 and FITC-dextran	PKH26 and FITC-dextran	60% proliferating cells (counted 90 dpt).	(Pšenička et al., 2015)

For cryopreservation and transplantation studies, purification phase of early stage germ cells is necessary. Primordial germ cells (PGCs) oogonia (OG) or spermatogonia (SG) must be allocated from other testicular cells like spermatids and spermatozoa before further applications. Recently there are several techniques of germ cell isolation; cell sorting via flow cytometry (Kobayashi et al., 2004), magnet-activated cell sorting (von Schönfeldt et al., 1999), density gradient centrifugation-elutriation (Loir and Le Gac, 1994) and in fish species simple and economic method Percoll gradient is used (Lacerda et al., 2006, Pšenička et al., 2015). But none of them has been tested on pikeperch. I applied enzyme dissociation and Percoll solution sorting technique for isolation of pikeperch (*Sander lucioperca*) early stage germ cell.

1.1 Germ Plasm Development

Germ cells are formed as primordial germ cells (PGCs) early in embryonic development in fishes as in many other organisms. Generation, migration, proliferation, and differentiation of PGCs and production of gametes have been examined in reproduction of organisms. In several studies, nematode *C. elegans*, fruit fly *Drosophila*, frog *Xenopus* and zebrafish *Danio* have been preferred as model animals (Richardson and Lehmann, 2010, Strome and Lehmann, 2007). They have focused on PGCs during early embryogenesis in these organisms. Maternal factors are usually concentrated in germinal granules in oocytes and cleavage embryos (Strome and Lehmann, 2007). Maternally provided-cytoplasm that contains factors directly involved in the germ cell fate decision is called germ plasm (GP). Asymmetrically-localized GP, or germ plasm-like structures that are visible under transmission electron microscopy (TEM), which are called “nuage”, are found in positions where putative PGCs (PGCs) are formed. They have proved that the cytoplasmic substances are responsible for inducing PGC development (Herpin et al., 2007a, 2007b, Strome and Lehmann, 2007, Yoon et al., 1997). In *Drosophila* it has been shown that the GP has an instructive role in directing nuclei to the germline (Illmensee and Mahowald, 1974). Zebrafish embryos from which the GP was removed lacked germ cells (Hashimoto et al., 2004). Also nuage have been identified by TEM in PGCs of teleosts (Bruslé and

Bruslé, 1978, Herpin et al., 2007a, Parmentier and Timmermans, 1985, Sàbat et al., 2009, Satoh, 1974, Schulz et al., 2010, Timmermans and Taverne, 1989, Vázquez et al., 2012). In zebrafish, DNA-helicase RNA (encoded by the *vasa* gene) becomes concentrated along cleavage planes at the two-cell stage of embryogenesis, and remains exclusively associated with four cells (putative PGCs) through development to the 1000-cell stage (Yoon et al., 1997).

In mammals and urodeles the mechanisms of germ-line specification are different. Consistently, the formation of the founding population of PGCs in the mouse was shown to depend on cell-cell interactions by extracellular factors of the bone morphogenetic protein (BMP) family (Tsang et al., 2001, Ying and Zhao, 2001). Whereas the actual specification and formation of PGCs in mammals and in urodeles appear to be independent of GP, material resembling nuage, a GP organelle, is found in germ cells of these organisms at later developmental stages (Chuma et al., 2009).

1.2 PGC Specification

There are two mechanisms to establish the germ cell lineage in the embryo. The first way is called preformistic like in *C. elegans*, *Drosophila*, *Xenopus* and zebrafish and involves that the cells destined to become germ cells inherit the specific germ cell determinants present in the germ plasm. By tracing germ plasm, or nuage, the germ line can be followed as an independent cell lineage from the inception of development through adulthood. The second way is found in urodeles and mammals, where germ cells are not specified by such determinants but by signals controlled by zygotic genes. In mammals, a few cells of the early embryo are induced by signals of neighboring cells to become primordial germ cells (Johnson et al., 2003). The primordial germ cells of urodele amphibians (salamanders) have an apparently different origin. Moreover, there does not seem to be any particular localized “germ plasm” in salamander eggs. Rather, the interaction of the dorsal endoderm cells and animal hemisphere cells creates the conditions needed to form germ cells in the areas that involute through the ventrolateral lips. So in

salamanders, the PGCs are formed by induction within the mesodermal region and presumably follow a different path into the gonads (Gilbert, 2001). In *Drosophila* and *Caenorhabditis*, the germ line is separated from the somatic tissue immediately at the onset of embryogenesis, whereas, in *Xenopus*, restriction to the germ line occurs shortly before gastrulation. Nuage-like structures are detected during oogenesis and late embryogenesis, and the origin of germ cells has been traced to different germ layers in different fish (Herpin et al., 2007a). It is known that germ plasm is observed in several fishes and germ cell specification modes in amphibians and insects are diversified even between species of the same order (Peng et al., 2009).

Drosophila, zebrafish, and *C. elegans*, germ cells form in a specialized, maternally synthesized cytoplasm, which is thought to contain germ cell determinants (Soto-Suazo and Zorn, 2005). In *Drosophila*, PGCs first form as a small group of cells at the posterior midgut primordium of blastula. They are the first cells to cellularize after the arrival of nuclei at the end of the syncytial blastula stage. The essential triggers for their cellularization are not nuclei, but centrioles (Starz-Gaiano and Lehmann, 2001, Wylie, 1999). This process requires the activity of several germ plasm specific RNAs and proteins. In particular, three germ plasm localized RNAs, *germ cell-less* (*gcl*), *nanos* (*nos*) and *polar granule component* have been implicated in the early events of germ cell specification, although only *gcl* seems to be directly required for PGC formation (Li et al., 2006, Santos and Lehmann, 2004). Zebrafish PGCs also form during early embryogenesis around 3 hours, PGCs do not form at a single embryonic position. Instead, four PGC clusters, each containing approximately four cells, form at random locations in the early embryo (Raz, 2004, Yoon et al., 1997). The zebrafish homolog of the *vasa* gene has made it possible to analyze germ cell formation in zebrafish embryos. As in *Drosophila*, *vasa* RNA is supplied maternally and is contained within electron-dense nuage particles distributed throughout the one cell stage embryo. However, unlike in other organisms, where germ plasm is localized to one particular region of the egg (the posterior pole in Drosophila or *C. elegans*, or the vegetal pole in *Xenopus*), *vasa* RNA in the zebrafish localizes to the edges of the cleavage planes in two and four cell embryos and segregates asymmetrically during

subsequent divisions (Braat et al., 1999, Doitsidou et al., 2002, Knaut et al., 2000, Olsen et al., 1997, Yoon et al., 1997). Through asymmetric division, only four germ cell precursors containing *vasa* RNA are clearly separated from the somatic cells. At the sphere stage (cycle 13), *vasa* containing cells begin to divide symmetrically to give rise to four germ cell clusters that are positioned independently of the embryonic axis. Inheritance of maternal *vasa* RNA seems to be a good indicator of germ cell fate, since zygotic *vasa* RNA transcription and protein translation are initiated only in the four germ cell clusters and are accompanied by nuage particles. Furthermore, analysis of *vasa* gene knockdown in embryos shows that cells maintain `germ cell morphology' even loss of *vasa* mRNA expression (Knaut et al., 2000, Weidinger et al., 1999). In contrast to Drosophila, zygotic transcription is activated in zebrafish at the same stage in germ cells and the soma (Richardson and Lehmann, 2010, Starz-Gaiano and Lehmann, 2001).

Two other genes, *piwi* (Li et al., 2012), *H1M* (Müller et al., 2002, Wibrand and Olsen, 2002) and *dead end* (Weidinger et al., 2003), are involved in germ cell formation in zebrafish. *piwi* piRNAs are identified from Drosophila to vertebrates such as *Xenopus*, zebrafish, madaka also mice as a gene involved in determining and specification of PGCs (Li et al., 2012). *H1M* mRNA is identified in *Xenopus* and homologue in mice, restricted to PGCs after gastrulation. *dead end* mRNA is localized maternally at the ends of cleavage furrows at the 4-cell stage, as are *vasa* and *nanos*1 in zebrafish., beyond that electron microscopy has revealed that germinal granule-like structures are localized near the indented furrows at the 4-cell stage, and *vasa* transcripts are embedded in these structures (Knaut et al., 2000). Based on these morphological observations, the cytoplasm at the distal ends of cleavage furrows is considered to contain germ lineage determinants in zebrafish embryos, although the requirement of the cytoplasm for germ cell formation is not yet clear (Hashimoto et al., 2004).

Although deep studies in model animals and fishes as shown above, overview of PGCs specification in fishes is still unclear. In early studies on PGC in fish, PGCs were identified only by their morphological features because they have features that are distinct

from somatic cells. PGCs are round and larger than other cells with large nuclei, and possess prominent nucleoli and distinct nuclear membrane. Observation in several fish, PGC morphology data were identified by light microscope has been reported by many researchers (Braat et al., 1999, Gamo, 1961, Gevers, 1992, Johnston, 1951, Nedelea and Steopoe, 1970). In medaka, PGCs were first recognized during early and mid-gastrula stages within ectoderm and mesoderm and during neurula stages within endoderm (Gamo, 1961). In carp, PGCs were first recognized during late gastrulation among mesodermal derivatives (Nedelea and Steopoe, 1970). In Micropterus salmoides, Johnston (1951) observed PGCs in the posterior hypoblast during gastrulation. In Barbus conchonius, PGCs were first identified at 100% epiboly, within the mesoderm. Subsequently, during early somitogenesis PGCs were found in the peripheral endoderm (Gevers, 1992). Richards and Thompson (1921) also identified PGCs in the peripheral endoderm of Fundulus heteroclitus embryos during early somitogenesis.

Nowadays there are more sophisticated ways for much earlier identification of PGCs which are in situ hybridization (ISH) and green fluorescent protein (GFP) labelling technology (Farrell, 2011). ISH is a powerful technique that allows for specific localization of nucleic acid targets in fixed tissues, cells or tumors (Yu et al., 2013). For hybridization, sample cells and tissues are treated to fix the target transcripts in place and to increase access of the probe which can be labeled by complementary DNA or, now most commonly, a complementary RNA (riboprobe) (Farrell, 2011, Jin and Lloyd, 1997). In zebrafish, PGCs had been identified by using vasa riboprobe. Their distinct morphology at the 5 somite stage in the peripheral endoderm in association with the YSL, whereas they identified PGCs within the hypoblast already during mid gastrulation (Braat et al., 1999). The probe hybridizes to the target sequence at elevated temperature, and labeled with either radio-, antigen-labeled or fluorescent- bases like GFP. On GFP labelling method identification can be made by establishing a transgenic strain that contains GFP gene regulated by PGC-specific regulatory sequences or injection of mRNA containing a GFP-coding sequence and PGC-specific untranslated region (UTR) (Farrell, 2011). Solution parameters such as

temperature, salt, and/or detergent concentration can be manipulated to remove any non-identical interactions (Jin and Lloyd, 1997).

1.3 PGC Migration

As described in germ plasm development, *Drosophila* and zebrafish require germ plasm a specialized cytoplasm that contains maternal RNAs and proteins. In the *C. elegans* embryo, PGCs also form in germ plasm and much is known about their specification, but *C. elegans* PGCs do not show a certain migration and seem to reach the gonad by ingression during gastrulation (Nakamura and Seydoux, 2008).

PGCs of *Drosophila* display migratory behaviors during gastrulation and they are carried by tissue movement into the forming posterior midgut pocket of the embryo. In the posterior midgut, PGCs form a tight cluster with each other in the lumen but make little contact with the surrounding somatic cells. PGC cluster takes on a characteristic radial organization, with the leading edge of each cell facing outwards towards the posterior midgut take signal by Trapped in endoderm 1 (TRE1). Subsequently, PGCs begin to extend cellular protrusions towards the surrounding posterior midgut cells and lose adhesion with each other. Active PGC migration begins as the cells disperse from the cluster and individually migrate through the posterior midgut. Tre1 not only leads to the polarization of PGCs, but also carries out additional functions in mediating the directed migration of PGCs by inducing adherent components as E-cadherin, RHO1 and Gβ (Kunwar et al., 2006). Wunen (WUN) and WUN2 regulate migration into the mesoderm. WUN and WUN2 are expressed at sites that PGCs avoid, such as the ventral midgut, and in PGCs. Data suggest that WUN and WUN2 hydrolyse an extracellular phospholipid that functions as a PGC attractant and survival factor. PGCs are attracted to somatic gonad precursors (SGPs) by the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) pathway, which adds a geranyl-geranyl (GG) group to a putative chemoattractant. Multidrug resistance 49 (MDR49), an ABC transporter, is required for chemoattractant secretion (Richardson and Lehmann, 2010).

In the mouse, PGCs first appear in 7 day old embryos in both early pregastrulation and the early-streak stage as a cluster (Ginsburg et al., 1990). In 8.5 day old embryos, they move into the hindgut epithelium. This is a rather efficient means of transportation for the PGCs and has the advantage of placing them in close proximity to the regions where the gonadal ridges will soon form. From this location, the PGCs move through the mesenchyme of the dorsal mesentery toward the genital ridges, where they later differentiate into either oogonia or spermatogonia. PGCs have a smooth, round morphology at 8.0 - 8.5 day. Most are still embedded in the epithelium of the developing hindgut during gastrulation. PGCs acquire a polarized morphology before initiating their migration into the endoderm. During this migration, the PGCs proliferation increases from less than 100 to approximately 4000 during the period of migration (Soto-Suazo and Zorn, 2005). Although, the molecular mechanisms regulating this polarization are not understood, it is thought that PGC migration regulated by interferon induced Transmembrane protein 1 (IFITM1). The receptor tyrosine kinase (RTK) ckit is required for general PGC motility, although it seems that PGCs are still capable of initiating migration when this pathway is disrupted. PGC migration to the genital ridges is also controlled by CXCR4 and SDF1 in mice. SDF1 is expressed by the somatic cells of the genital ridge and PGCs express CXCR4. Integrin β1 is also required for this step. PGC motility and survival requires the receptor tyrosine kinase c-kit (also known as KIT) and its ligand steel (also known as KITLG). Steel is expressed by somatic cells surrounding PGCs throughout migration (Richardson and Lehmann, 2010).

During *Xenopus* cleavage, germ plasm material is brought upward through the yolky cytoplasm, and eventually becomes associated with the endodermal cells lining the floor of the blastocoel (Kloc et al., 1993). The PGCs become concentrated in the posterior region of the larval gut, and as the abdominal cavity forms, they migrate along the dorsal side of the gut, first along the dorsal mesentery (which connects the gut to the region where the mesodermal organs are forming) and then along the abdominal wall and into the genital ridges. They migrate up this tissue until they reach the developing gonads. *Xenopus* PGCs

move by extruding a single filopodium and then streaming their yolky cytoplasm into that filopodium while retracting their "tail." Contact guidance in this migration seems likely, as both the PGCs and the extracellular matrix over which they migrate are oriented in the direction of migration (Wylie, 1999). Furthermore, PGC adhesion and migration can be inhibited if the mesentery is treated with antibodies against *Xenopus* fibronectin (Heasman et al., 1981). Thus, the pathway for germ cell migration in these frogs appears to be composed of an oriented fibronectin-containing extracellular matrix. The fibrils over which the PGCs travel lose this polarity soon after migration has ended. As they migrate, *Xenopus* PGCs divide about three times, and approximately 30 PGCs colonize the gonads (Whitington and Dixon, 1975). These will divide to form the germ cells.

Although the details of the mechanism and pathway of PGCs migration in teleosts have yet to be clarified, migration of PGCs has been well investigated in the zebrafish (*Danio rerio*), and the medaka (*Oryzias latipes*) (Braat et al., 1999, Doitsidou et al., 2002, Weidinger et al., 2002, Yoon et al., 1997). In zebrafish, at specification, PGCs have a smooth, round morphology and do not possess migratory activity 3 hours after fertilization. PGCs begin to randomly extend small cellular protrusions in multiple directions at 3.5 hours after fertilization. These protrusions disappear during mitosis. At 4.5 hours PGCs become polarized and individualize and extend broad protrusions at the leading edge. This step is dependent on transcription and the *dead end* protein (Weidinger et al., 2003), and is necessary for the cells to respond to stromal-derived factor 1a (SDF1A; also known as CXCL12A) chemokine signaling (Doitsidou et al., 2002, Knaut et al., 2003). Also *de novo* transcription induces to initiate PGCs migration in zebrafish (Blaser et al., 2005). Zebrafish PGCs are sequentially transfer to the anterior and lateral boundaries of the mesoderm during gastrulation, move toward an intermediate region bordering the mesoderm during somitogenesis, become bilaterally aligned at 24 hours after fertilization to the anterior yolk extension and finally reach the gonad where PGCs coalesce with somatic cells into an intact gonad (Weidinger et al., 1999). More recent studies have shown that the expression of PGC specific proteins such as *vasa, nanos1* (Weidinger et al., 1999), *hmgcr, quemao (qm), ggt1* (Thorpe et al., 2004), *staufen* (Ramasamy et al., 2006), *Pik3* (Raz and

Reichman-Fried, 2006, Soto-Suazo and Zorn, 2005), *cyclopamine*, *gai*, *puf-A*, *Igf*, (Xu et al., 2010) containing proteins responsible for migration of PGCs. The zebrafish homologue of the Drosophila *piwi* is identified as *ziwi* responsible for migration of PGCs (Tan et al., 2002). A similar approach has been performed on medaka and *opiwi* identified as essential for PGC development and migration (Li et al., 2012).

In zebrafish, PGCs expressing the G protein-coupled receptor (GPCR) chemokine (CXC motif) receptor 4b (CXCR4B) migrate towards the CXCR4B ligand, stromal derived factor 1a (SDF1A; also known as CXCL12), which is secreted by somatic cells. Another somatically expressed GPCR, CXCR7B, promotes the internalization and degradation of SDF1A, which might lead to proper gradient formation and precise targeting of PGCs. Following PGC migration to an intermediate target, a new group of distant somatic cells begin to express SDF1A, directing PGCs to new targets (Richardson and Lehmann, 2010).

1.4 Sex Determination and Differentiation

Specific sex differences begin to appear after PGCs migration. Migration stops when PGCs reach the gonad and they begin to carry out their functions as germ cells by acquiring sex specific morphologies. A subset of germ cells in the gonad acquire the ability to function as germline stem cells, which undergo meiosis to produce sperm and eggs and promote the next generation of embryonic development and PGC migration (Richardson and Lehmann, 2010).

Male germ cell differentiation occurs continuously in the seminiferous tubules of the testes throughout the life of a normal animal. Spermatogenic cells at different stages are classified into four broad categories as spermatogonia, spermatocytes, spermatids, and spermatozoa with numerous substages defined within each category. All premeiotic cells are called spermatogonia; these include regenerating stem cells as well as those that have taken the path to terminal differentiation. With the commencement of meiosis, germ

cells are called spermatocytes, and subsequent to meiosis, haploid cells are called spermatids. Finally, with the release of the morphologically mature product, the germ cells are called spermatozoa or, more simply, just sperm. The timing of the stages of spermatogenesis in the mouse was described initially (Oakberg, 1956). At the beginning of spermatogenesis, the testicular tissue contains only undifferentiated type A1 spermatogonia, which will serve as a self-renewable stem cell population in course of the life of a male mouse. By third day, differentiation has begun through a series of mitotic divisions into more developed spermatogonial stages (Nebel et al., 1961). Functionally, type A spermatogonia are divided in two main group as undifferentiated (A single, As; A paired, Apr; A aligned, Aal; the latter usually clones of 4, 8, or 16 cells) that are constantly present; and in differentiated spermatogonia (A1–A4) that are present only some stages of the epithelial cycle (Schulz et al., 2010). By 8 to 10 days after starting of spermatogenesis, primary spermatocytes are frequently found in testis sections, which reflect the long duration of the first meiotic prophase. They can be identified easily by their relatively large size, and by the characteristic nuclear staining patterns, representing the progressively condensing chromosomes. During the first meiotic division, homologous (maternal and paternal) chromosomes become separated into the nuclei of secondary spermatocytes (Nebel et al., 1961, Schulz et al., 2010). The first round spermatids, are not seen until after twenty days. During the next 13 days, the round spermatids differentiate into elongating spermatids in which the nucleus condenses and the sperm tail forms. Finishing part of the process, morphologically-mature sperm are released into the fluid-filled lumen (Nebel et al., 1961). The whole process called spermatogenesis as differentiation from stem cell to released spermatozoa. The term spermiogenesis means particularly to the last morphological differentiation of haploid cells into sperm.

In fish, oogenetic cells at different stages are classified into four general categories like oogenetic cells which are; oogonia, oocyte, ootid (immature ovum), ovum. Significant steps of oogenesis include formation of PGCs into oogonia and followed by their transformation into primary oocytes, with the onset of meiosis. Following this, the development of the oocyte continues with vitellogenesis. During vitellogenesis the oocyte

gather nutritions needed for embryo after fertilization. At this period, the oocyte also accumulates maternal RNA and completes the differentiation of its cellular and non-cellular envelopes. After first meiotic division two cells occur in different sizes, the small cell with first polar body degenerates and the large secondary oocyte is formed (Lubzens et al., 2010, Schmerler and Wessel, 2011). Finally ovulation takes place at the end of the maturation. The secondary oocyte is then realized from its surrounding follicular cell layers and moves into the ovarian lumen or abdominal cavity. At this stage the female gamete is first known as ootid and after full maturation called ovum. Ovum is haploid as a result of the second meiotic division and second polar body which formed during this process also degenerates (Lubzens et al., 2010). Female germ cell differentiation operates under a two phase time course as undifferentiated gonad type and differentiated gonad type. In the undifferentiated gonadal type, the undifferentiated gonads differentiate into either testis or ovary, after once all gonads develop ovaries. In the differentiated type, the undifferentiated gonads directly differentiate into testis or ovary. The undifferentiated type of gonads are seen in the minnow *Phoxinus phoxinus* and the guppy *Poecilia reticulate* and the differentiated type of gonads are seen in the medaka the Atlantic halibut *Hippoglossus hippoglossus* and the rosy barb *Puntius conchonius* (Hayakawa and Kobayashi, 2012). On the other hand, ovarian differentiation commonly begins prior to testicular differentiation in most gonochoristic teleosts (Paul-Prasanth et al., 2011). For example, in the rosy barb, a differentiated-gonad type fish, the male gonad appears about 20 days after forming the female gonad (Çek, 2006). In *Colisa lalia*, sex differentiation of gonads is thought to occur between 25–30 days after hatching (Hayakawa and Kobayashi, 2012).

In medaka, the first symptom of the sex differentiation of fish is the difference in the proliferative activity of germ cells between males and females. About 40 PGC occur in endoderm and they hardly proliferate during the formation of gonads. There are no sexual differences in the number of PGCs in the gonads newly formed. The PGCs resume their mitotic activity immediately after the completion of gonad formation when female germ cell proliferates more actively then the males. Consequently, presumptive ovaries in the fry just after hatching contain about 200 germ cells, while presumptive testes about 80. Female germ cells (oogonia) continue to proliferate after hatching, but proliferation of male germ

cells (prespermatogonia) is arrested after hatching. After 10-15 days of a mitotically dormant stage, prespermatogonia resume their mitotic activity and begin to increase in number (Hamaguchi, 1992, Noriyuki Satoh, Egami, 1972). Satoh, (1974) has been reported that after the time of hatching, no essential changes are detectable with the electron microscope in the testis during 10 days of normal development. And it has been answered what mechanism is involved in sex differentiation in the natural course of development in this fish, the possibility that some intracellular mechanisms in the germ cell itself might take part in sex differentiation (Satoh, 1974).

Sex differentiation refers to the expression of phenotypic attributes specific to the sex of an individual. It is known that gonad development is a result of the presence or absence of the sex determination gene SRY on the Y chromosome in mammals. Sex differentiation is determined by the hormonal products produced by the gonads (Kurita and Sakai, 2004). Although, estrogen and 11-ketotestesteron play a role in regulating germ cell development, the mRNA expression pattern of *sox9* and *cyp19* during sex reversal are important factors to initiate and maintain masculinization or their elevation triggered male-to-female change (Guiguen et al., 2010). Although, sex chromosomes showed different origins and the sex-determining genes might differ in same genus (Li et al., 2011), sex determination associated genes such as *wt1*, *sf-1, amh, dax1*, *foxl2, sox9, gata*, *dmy*, *dmrt1, cyp19, and cyp21* direct and indirect regulatory effects on sex differentiating signal pathways in several fish species such as zebrafish, medaka, carp. *sf-1, gata and wt1* are responsible for the proliferation, differentiation of spermatogonia. Homologues of *sf-1* have been identified as *ff1d* is expressed in the hypothalamus and gonads, especially in testicular Sertoli cells and Leydig cells, and can regulate the expression of *amh* in zebrafish (Chen et al., 2013). Also it has been reported CA15b and β-actin may be necessary for PGC development transform in ovary in zebrafish (Wang et al., 2013). The mammalian homologues are specifically expressed in the adult testis and are not detectable in other tissues, including the ovary. In the mouse and human testes, *miwi* and *hiwi* expression is germline specific and is localized to the spermatocytes only (Tan et al., 2002). *Zili*, one of the two *piwi* proteins in zebrafish, has been reported that has been involved in PGC

maintenance and migration, and also germ cell differentiation in germ line meiosis (Houwing et al., 2008). Although genes that determine the development of the male or female sex are known in *C. elegans*, *Drosophila*, and most mammals, the identity of sex determining factors in several species remains unknown. According to results of the cloning the medaka sex determining genes found specific region on Y chromosome and has been identified as *DMRT1Y*. These genes expressed during male embryonic and larval development and in Sertoli cells of the adult testes (Nanda et al., 2002).

1.5 Germ cell transplantation

The development of a germ cell transplantation technique was an important moment in the germline studies. First successful reported instance of the use of this technology, PGCs were transferred to the blood stream of developing chicken embryos, produced germline chimeras and, after maturity, they were able to propagate donor-derived offspring (Tajima et al., 1993). Germ cell transplantation is the only convenient method available for investigation of spermatogonial stem cell life cycle. In this powerful and affective technique developed by Brinster and collaborators (Brinster and Zimmermann, 1994). Transplanted mice spermatogonia were able to colonize and develop in recipient mice testes and form fertile sperm with the donor genotype characteristics. Then, a similar method was developed in fish using transplantation of primordial germ cells carrying green fluorescent protein (GFP) into the coelomatic cavity of rainbow trout (*Onchorhynchus mykiss*) larvae (Takeuchi et al., 2003). After transplantation, these cells were able to migrate to and to colonize the undifferentiated gonad, giving rise to female or male germ cells depending on the individual's genetic gender, gonad had differentiated into an ovary or a testis. Furthermore, males taken from this transplantation were also able to produce sperm with donor genetic characteristics (Takeuchi et al., 2003). Using the same process, xenogenic germ cell transplantation among rainbow trout(*Onchorhynchus mykiss*) and masu salmon (*Onchorhynchus masou*) were also successfully achieved (Takeuchi et al., 2004). Okutsu et al. (2006) have also shown oocyte production from spermatogonia transplanted into the coelomatic cavity of rainbow trout larvae, and this result illustrates the plasticity and bipotential capability of spermatogonial cells (early germ cells) in fish.

According to age of host species we can divide all germ cell transplantation studies in three broad group as embryo transplantation, newly hatched fish larvae transplantation and adult fish transplantation (Lacerda et al., 2013).

1.5.1 PGC transplantation in fish embryos

Varied approaches to the production of germline chimeras in teleosts have been described. One of these methods consists of the intra-specific transplantation of PGCs into blastula-stage fish embryos. Through micromanipulation, PGCs are harvested from donor embryos and transplanted into blastula-stage recipients that have had endogenous PGC development blocked by the injection of a dead end antigen morpholino. This technique, which resulted as complete replacement of the recipient germline with donor PGCs, has been successfully applied in zebrafish (*Danio rerio*) (Ciruna et al., 2002, Giraldez et al., 2005) and was confirmed by the observation of F1 offspring generation showing donor-derived characteristics. Another method of xenogenic transplantation of PGCs has been developed by Saito et al. (2008), who demonstrated that a single pearl danio (*Danio albolineatus*) PGC microinjected into the blastodisc of blastula-stage zebrafish giving rise to both sex chimeras which produces only donor gametes (Saito et al., 2008).

1.5.2 Germ cell transplantation in newly hatched fish larvae

The production of a transgenic trout model carrying the green fluorescent protein (Gfp) gene driven by vasa gene promoter (a specific marker of germline cells) was the commencement point that allowed for the isolation of live PGCs for utilize in *in vitro* and *in vivo* studies, including studies of germ cell transplantation into newly hatched fish larvae (Takeuchi et al., 2002, Yoshizaki et al., 2002). In this approach of germ cell transplantation, isolated donor germ cells were transplanted into the peritoneal cavities of newly hatched larvae in a relatively simple and fast application which used a microinjector (Yoshizaki et al., 2011). Newly hatched larvae were chosen because they did not possess a functional immune system, as indicated by the lack of both their thymus and their T cells. Lack of any

active immune system allowed the immunorejection of donor-derived germ cells to be avoided.

In the first reported studies of intraperitoneal transplantation of PGCs into hatchlings (Takeuchi et al., 2003) which used GFP-transgenic trout as the donor and wild-type trout as the recipient, transplanted PGCs were able to migrate toward and proliferate in the genital ridges of recipient larvae. In addition, donor-derived PGCs differentiated into mature eggs and sperm in the gonads and, resulting gametes which artificially fertilized produced normal offspring showing the donor-derived phenotype (Takeuchi et al., 2003). In order to develop a technology that would simplify the production of commercially valuable fish as well as the conservation of endangered species, PGC transplantation was performed in salmonids. Vasa-Gfp transgenic rainbow trout were used as donors, while masu salmon hatchlings were used as the host were able to produce xenogenic donor-derived offspring (Takeuchi et al., 2004). Okutsu et al. (2008) performed a spermatogonial transplantation between vasa-Gfp transgenic rainbow trout donors and sterile triploid masu salmon hatchling hosts. 2 years after transplantation, triploid salmon recipients produced only trout sperm and eggs (Okutsu et al., 2008).

1.5.3 Germ cell transplantation in adult fish

Lacerda et al. (2006) developed a recent, faster way to accomplish spermatogonial transplantation in Nile tilapia (*Oreochromis niloticus*), in which spermatogonia were transplanted through the urogenital papilla of adult fish. In the beginning of this method, the recipient fish had their spermatogenesis which suppressed by using busulfan (1,4-butanediol dimethanesulfonate). Afterwards type A spermatogonial cell suspension was obtained from donor testis and labeled with the fluorescent lipophilic dye (PKH26-GL) before being injected into the adult testes of the recipient through urogenital papilla. This method showed, sexually mature fish could be suitable as a recipient in germ cell transplantation. The recipient testes were analyzed after transplantation and showed PKH26-labeled germ cells in the lumen of the seminiferous tubules. Based on the

appearance and histology of the testis, germ cells did not appear to trigger immunorejection in adult allogenic recipient.

2. Material and Method

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

2.1 Fish

Three individuals were used for each 14 and 18 months old outdoor pond reared pikeperch (*Sander lucioperca*) taken from South Bohemian research center of aquaculture and biodiversity of hydrocenoses for this study.

2.2 Isolation of Gonad Cells by Enzymatic Dissociation

Immediately after killed three pikeperch males (126.62g / 26.73cm average body weight and average length), testicular tissue (0.513 g average weight) were dissected individually. 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, 304 mOsm/kg, pH 8) (Sigma-Aldrich, s.r.o., Prague, Czech Republic). Testicular Tissue was divided in two pieces and half of the testicular tissues were fixed in Bouin's solution (Sigma) for histologic examinations.

Second part was cut into small pieces, and transferred into 15 ml tubes with 10 ml of PBS + 0.3% trypsin (Sigma-Aldrich) (304 mOsm/kg, pH 8) used for enzymatic dissociation according to (Pšenička et al., 2015). 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 obtained suspension was filtered with 50 μm filter (Partec, Germany) to collect debris. To stop enzymatic digestion 1% BSA was added (Sigma-Aldrich A7511). 40 μg mL-1 DNAse (AppliChem A3778) was added to digest DNA from cells broken during dissociation. The tubes with gonad-cell suspension were centrifuged at 800 x g for 30 min at 4°C. The pellets were resuspended in 0.3 mL of media without enzymes.

2.3 Flow cytometry

After enzymatic dissociation of fresh testicular tissue, ploidy level was determined by flow cytometer (PARTEC Club 8, Münster, Germany) using a CYSTAIN DNA 1 steps kit (PARTEC). Briefly, one half of the enzymatically dissociated testicular tissue from each sample was diluted in 1.5 ml phosphate buffered saline (PBS, 304 mOsm/kg, pH 8). In second dilution, samples were diluted 30 times in PBS and numbers of cells were counted in 100μl.

2.4 Percoll Gradient

To separate early germ cell (eGC) from other testicular (spermatids and sperm) and somatic cells, a step Percoll gradient (Sigma-Aldrich) 5% and 33% was prepared. The testicular cell suspension was slowly transferred to the surface of the gradient and immediately centrifuged at 800 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 5% and 33% layers and the pellet (precipitate collected at the bottom of the tube), washed with 10 ml PBS (300 mOsm/kg, pH 8) and centrifuged at 800 g, 4°C for 30 min. 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). The eGC 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).

2.5 Histology

For histological observation of early and late germ cells, testicular tissue fixed with Bouin's fixative and stored in 80% ethanol until dehydration process for six days. Fixed samples were dehydrated using a Butanol series, and subsequently embedded in a paraffin block. Serial transverse sections of 5 μm thickness were stained with hematoxylin and eosin according to the standard procedures.

2.6 Cell Counting

In order to count cells, histologic sections and percoll gradient pictures were used whit random square. For each samples same square used as a template on pictures at three times for coverage of upper, middle and lower parts of sections and at least 1000 cells counted and distinguished by their size and shape according specific characteristics of early and late germ cells in different stages (Schulz et al., 2010). Early male germ cells identified easily by their relative size, and by the characteristic nuclear staining patterns, representing the progressively condensing chromosomes (Fig 2). The cells confirmed by light optical microscope Olympus BH2 (Olympus Corp., Tokyo, Japan) at 250× magnification and photographed by Nikon 5100 camera (Nikon, Tokyo, Japan).

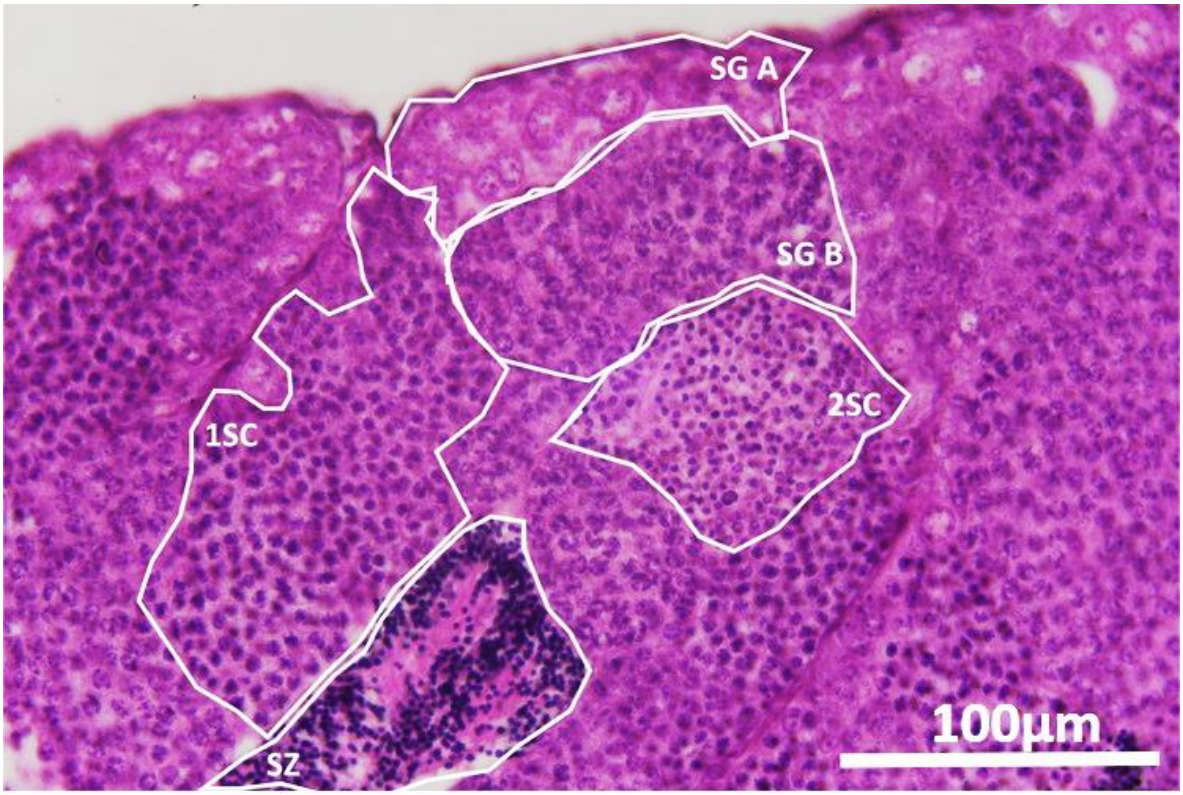


Figure 2. Pikeperch testis histologic section shows distinguished cells by their shape and size. Spermatogonia A (SG A), Spermatagonia B (SG B), primary Spermatocyte (1SC), secondary spermatocyte (2SC), spermatids and spermatozoa (SZ).

2.7 Statistical analysis

To compare the number of GC from different percoll gradient, one-way ANOVA and Tukey's test using Statistica for Windows, v. 9.1 (StatSoft, Inc., USA) were applied. Probability values of $P < 0.01$ were considered as significant.

3. Results

3.1 Adjustment of osmolality.

Blood samples osmolality (mean ± SD; n = 4) was 304 ± 2 mOsm/kg. The osmolality of the media used for experiments was adjusted to this level. Osmolality of blood samples was assessed using a Vapour Pressure Osmometer Vapro 5520 (Wescor, Logan, USA).

3.2 Adjustment of correct stage for isolation.

To evaluate the correct stage for spermatogonia isolation two different ages group of pikeperch were used in this study. Three male pikeperch were dissected for each age group. 14 month (Fig 3) and 18 month old pikeperch were examined histologically and total spermatogonia numbers in tissue were compared by random square counting and light microscope observation. 14 month old sample's average weight, length (87.7 g/ 22.1 cm) and gonad weight (0.0601 g) were recorded. 18 month old sample's average weight, length (126.62g / 26.73cm) and gonad weight (0.513 g) were also recorded for further comparison. 14 month old pikeperch samples were in early stage of maturation and showed average 98.4% early stage germ cell in testicular tissue. On the other hand 18 month old samples showed average 45.4% early stage germ cells. These two different age groups are both good candidates for spermatogonia isolation and transplantation. Fourteen months old pikeperch contained germ cells almost only in early stages and other isolation is not needed. On the other hand, the tissue as well as total number of germ cells in one testis is small. That is why i used 18 months old pikeperch (Fig 4) for other experiments, which testes contained higher number of early germ cells in total.

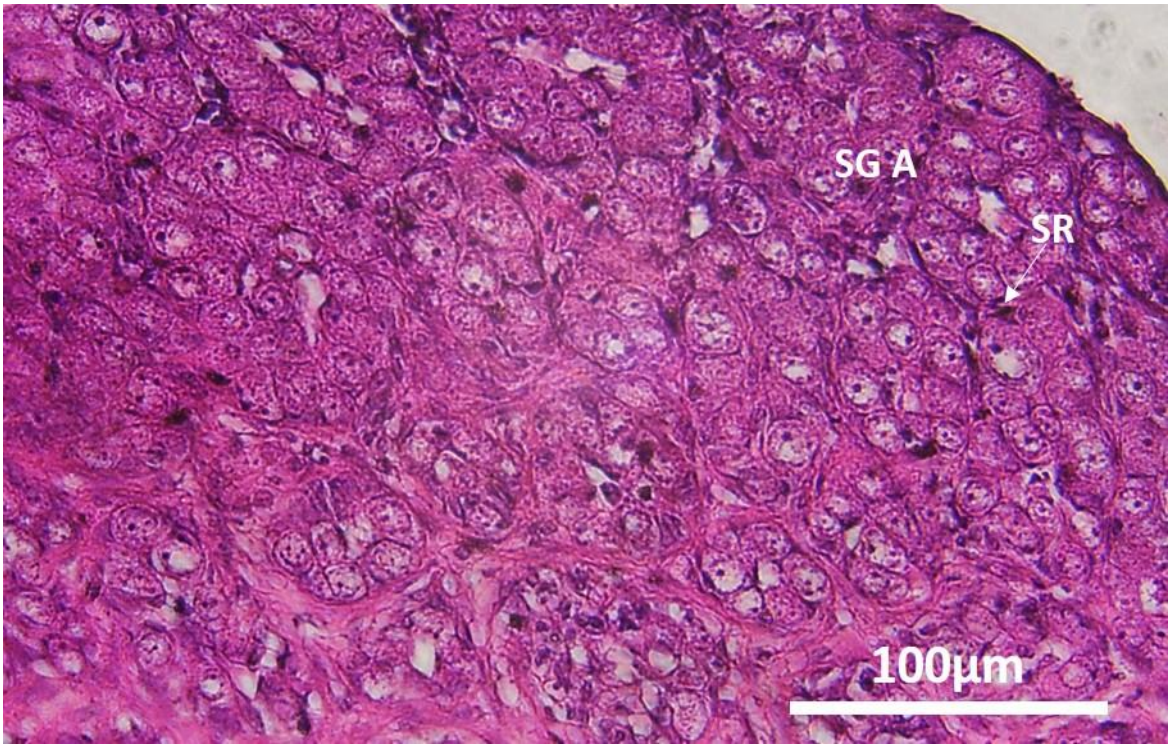


Figure 3. 14 month old pikeperch testis histologic section shows spermatogonia type a (SG A) and sertolli cells (SR).

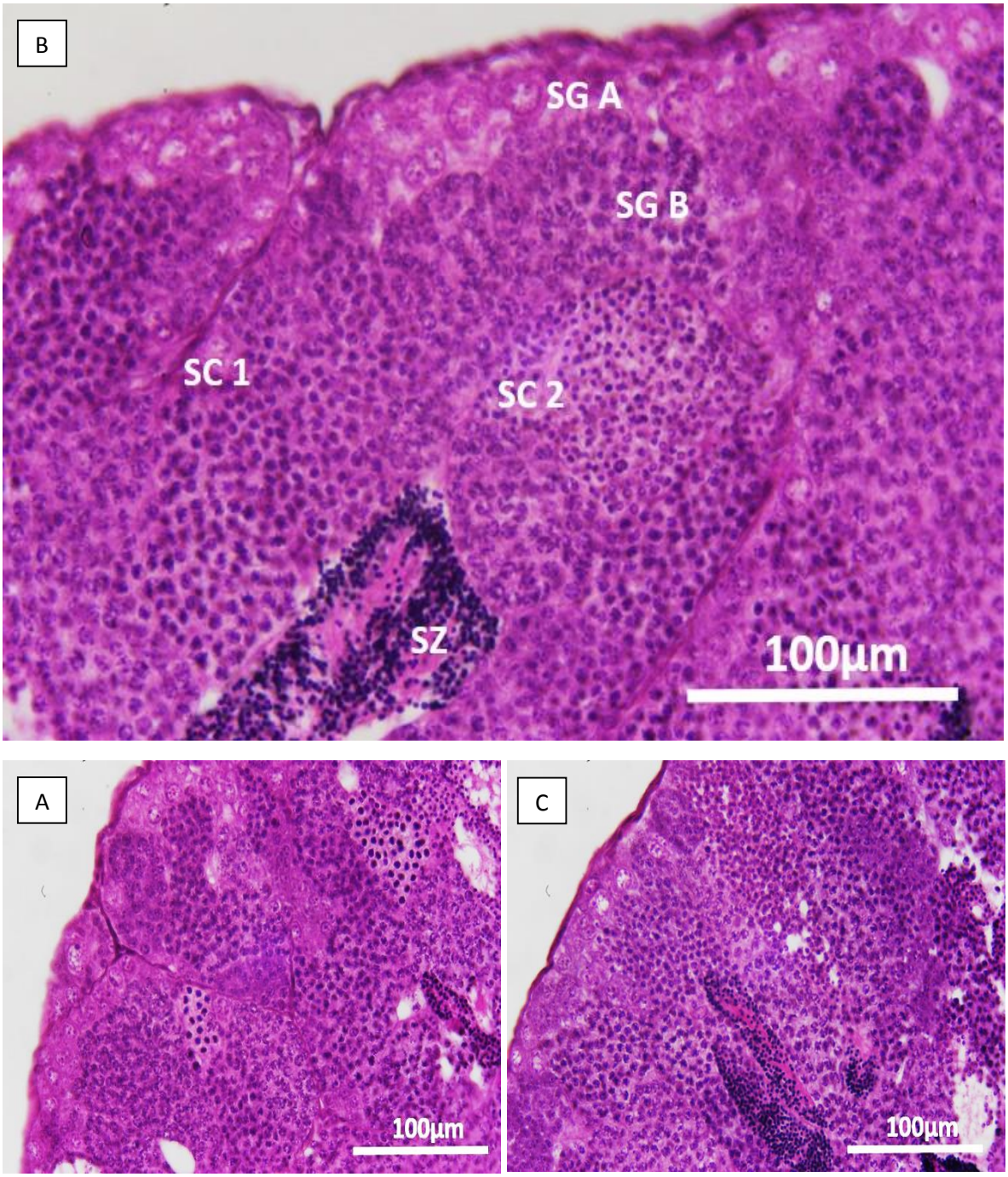
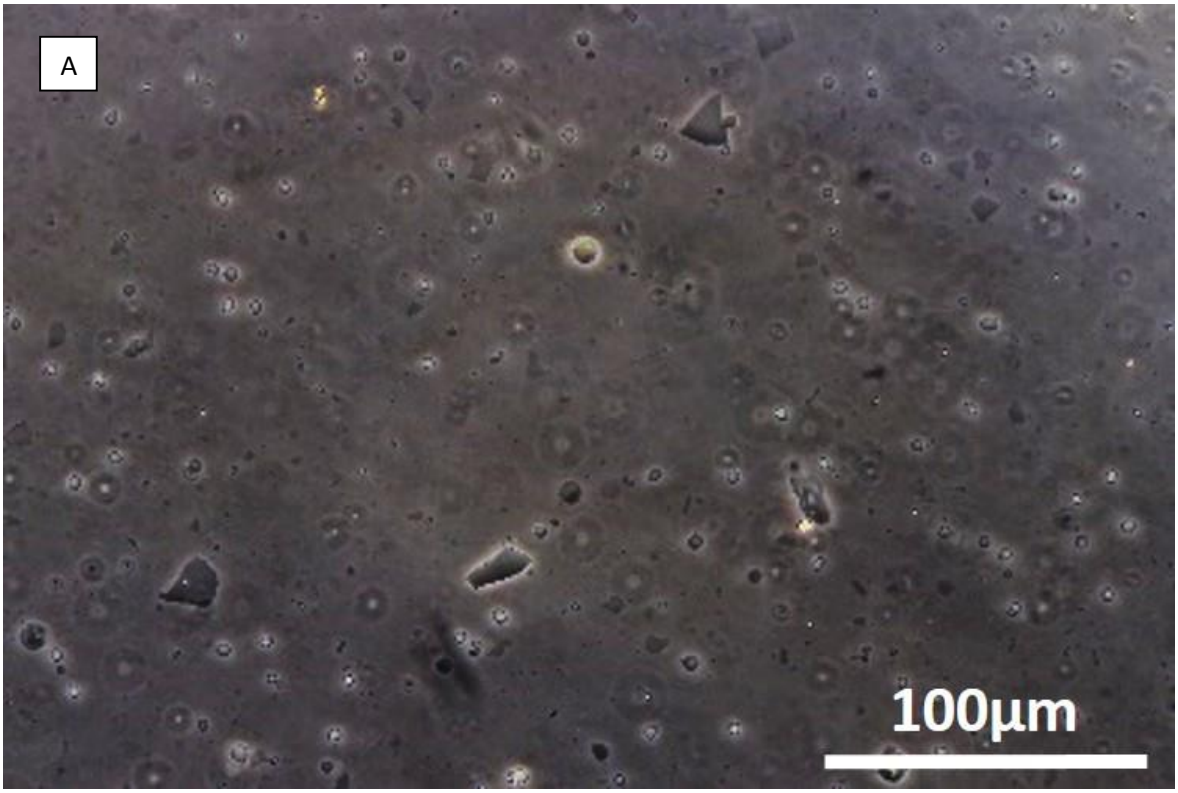


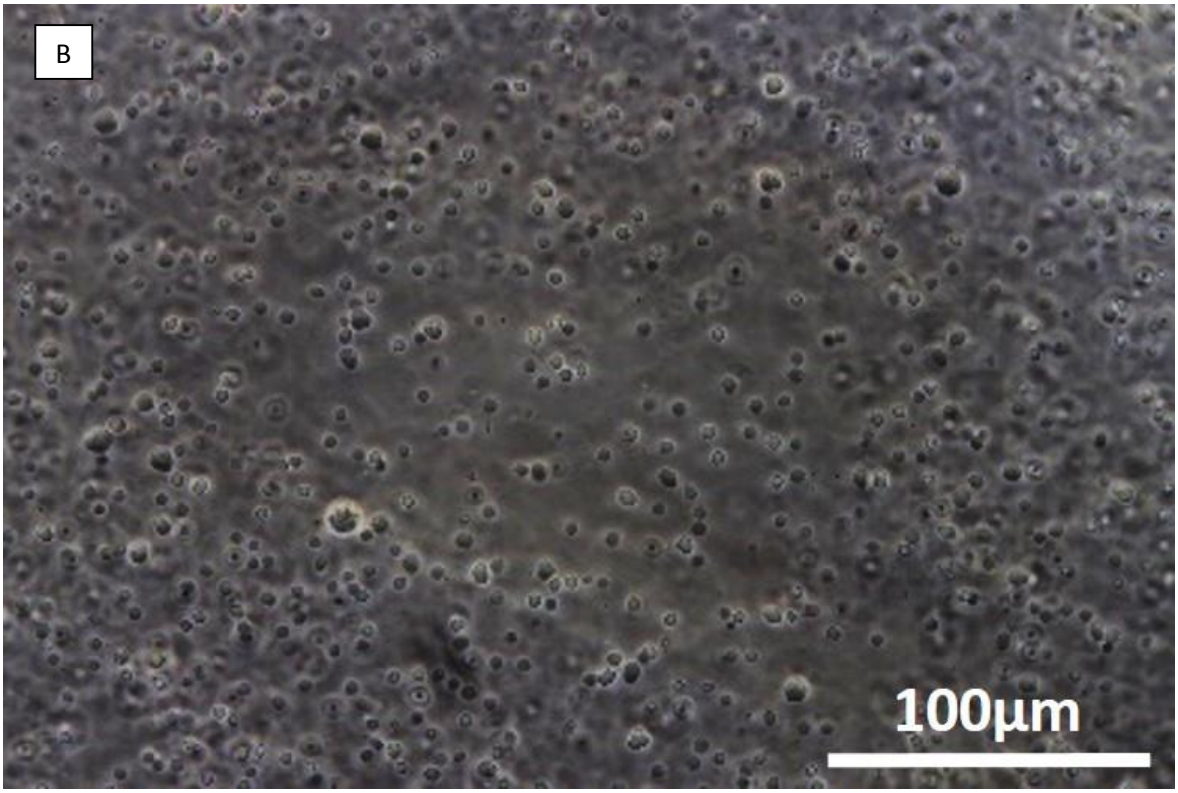
Figure 4. 18 month old pikeperch's testis histologic sections from each sample. (A) Sample 1 (B), Sample 2 (C) Sample 3.

3.3 Isolation of early stages germ cells in a Percoll gradient

The early stage germ cells 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 (Fig 5).

The percentage in the 5% and 33% layer and pellet of the percoll gradient from the three fish was calculated. The spermatogonia and spermatocytes were calculated together because of difficulty to distinguish from each other by light microscopy. Also spermatids were counted together with sperm because of visible smaller sizes than GC. The upper layer in the Percoll gradient (5%) showed 86% lipids + debris and 14% eGC. The 33% layer comprised 84.7% GC, 15.3% spermatids + spermatozoa. The pellet consisted of 1.57% eGC and 98.43% spermatids + spermatozoa. The percentage of eGC in each Percoll gradient layer were recalculated according to the total number of cells counted in whole percoll gradient (n = 3088). The percentage of eGCs were 0.84% in 5% layer, 41.58% in 33% layer, and 0.71% in pellet. Thus the highest concentration of GC was found in the 33% layer of Percoll gradient (Fig 6).


A
100µm


B
100µm

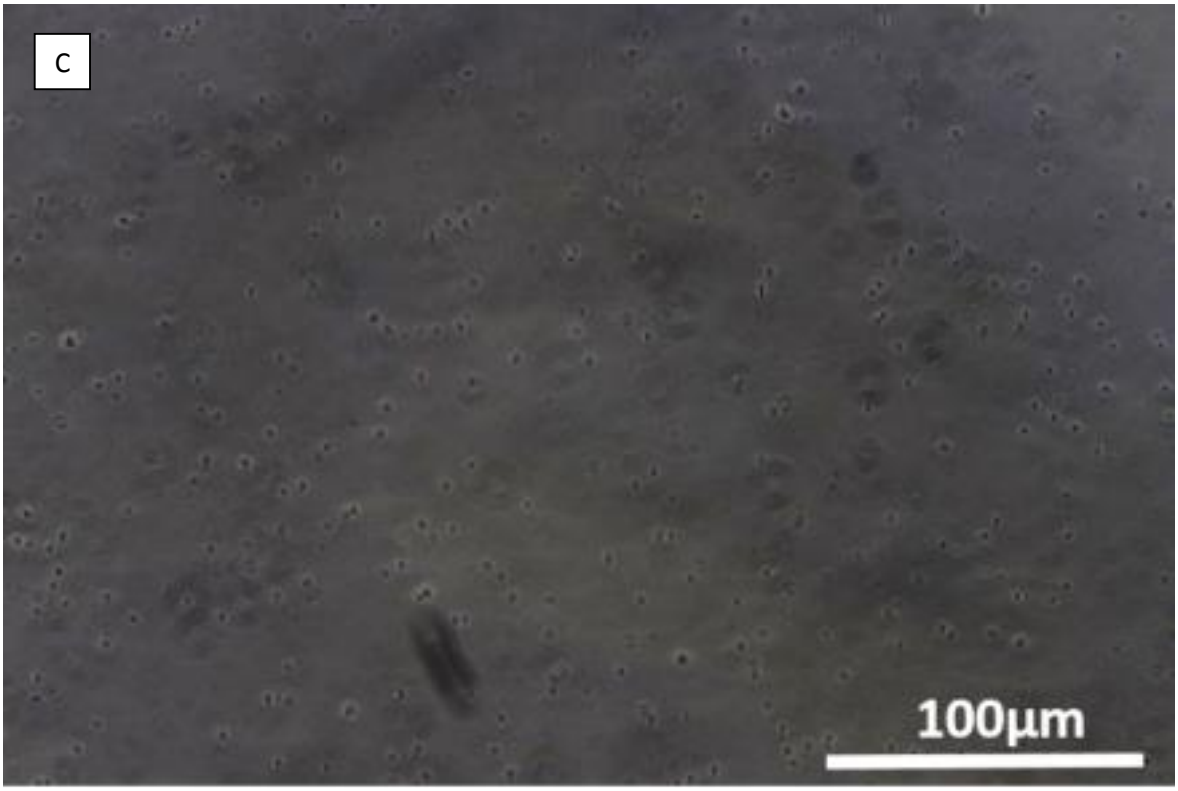


Figure 5. Pikeperch testicular and somatic cells of a single male separated by Percoll gradient 5% layer (**A**), 33% layer (**B**), and pellet (**C**) show lipid components with debris, early stages of germ cells, and spermatids with spermatozoa, respectively. All pictures taken in same magnification. Scale bar = 100 µm

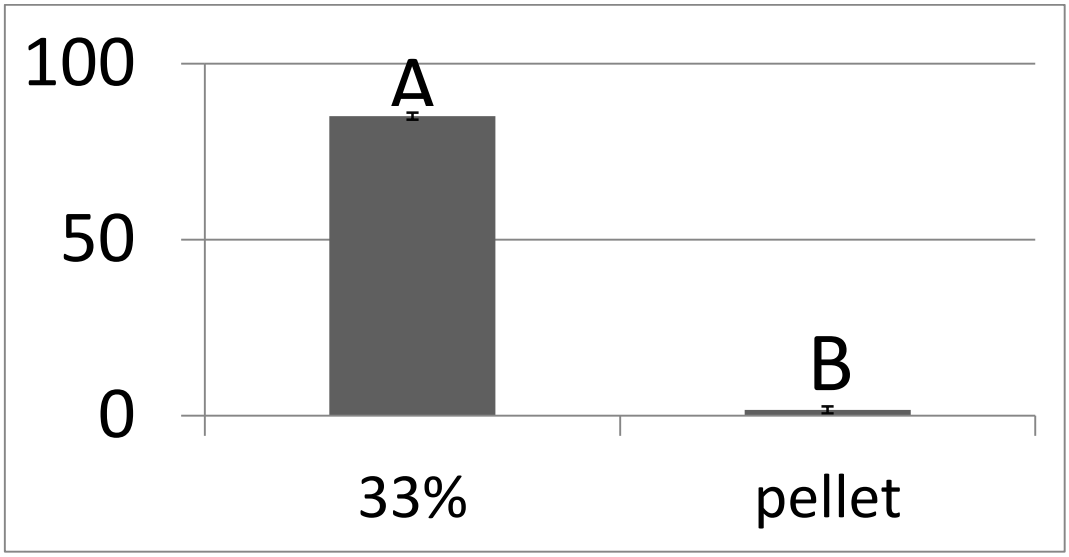


Figure 6. Graph shows percentage of early stage germ cells within the 33% percoll gradient (A) and pellet (B) are significantly different (ANOVA, $P < 0.0016$).

3.4 Flow cytometry

Beside the random square counting, flow cytometry was also used for determination the percentage of early and late stage germ cells depending on ploidy level (Table 2). After enzymatic dissociation of fresh testicular tissue, ploidy level was determined by flow cytometer. The number of haploid and diploid GC in each sample were recalculated depending 100 µl volume of cell suspension. The average number of haploid GCs in three samples was 8.334 and average diploid GCs were 11.633 in 100 µl.

Table 2. Yield of germ cells (GC) from testes (average volume 0,128g) of individual pikeperch obtained from cell suspension. Table shows haploid (n), diploid (2n) cell number depending on recalculation in 100 µl.

Sample no	n	2n
1	8.688	9.762
2	7.876	12.927
3	8.438	12.300

4. Discussion

According to studies made, the following can be suggested: (1) the Percoll gradient is an inexpensive, simple, and practical method of distinguish testicular cells with the highest percentage of early stage GC in the 33 % layer; (2) on pikeperch evaluate the correct stage of maturation is a key factor for successful spermatogonia isolation. This study showed natural pond reared 14 month old pikeperch has 98.4 % early stage GC in low volume (average 0.0601 g) of testicular tissue. On the other hand 18 month old samples which are kept in the same conditions showed 45.4% early stage germ cells in high volume (average 0.513) of testicular tissue. Depending on the purpose which can be spermatogonia isolation, cryopreservation or scientific study, each of these two age groups could be good candidates for early stage germ cell isolation. In addition i also would like to report that flow cytometry result for early and late stage cell counting depending ploidy level is not found reliable and not used as a result in this study. Moreover, i don't know whether flow cytometry method is worse or better than random square counting method. In my case it just showed significantly different result (Fig 7).

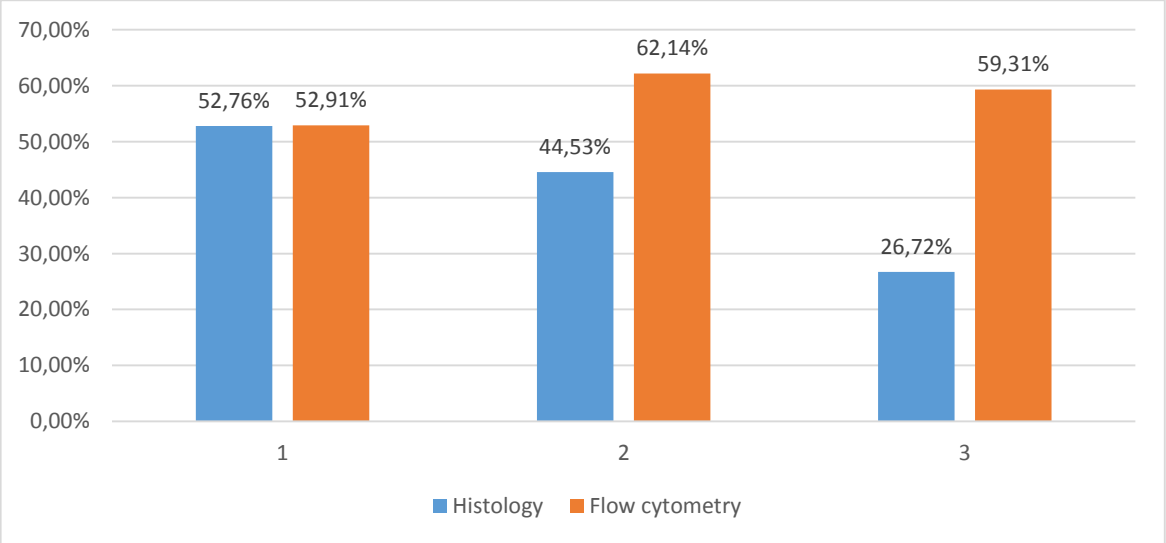


Figure 7. Graph shows difference between early germ cell percentages according to random square counts from histologic sections and flow cytometry counts for sample 1 (1), sample 2 (2) and sample 3 (3).

4.1. Importance of germ cells

Production of chimeric species through germ cell transplantation is a widening branch of bioengineering. This technique can be improve the gamete production of species which valuable, endangered or have complex reproduction systems by using more productive species or one already adapted to artificial reproduction as a host (Okutsu et al., 2006, Yamaha et al., 2007). Primordial germ cell has ability to migrate to genital ridge via chemotaxis. Relied to this characteristic PGC transplantation was developed and practiced intra and inter species (Saito et al., 2010, 2008). Despite this migration ability was proposed to be lacking in early stages of germ cells as SG/OG (Raz and Reichman-Fried, 2006) this argument has been refuted by several researchers (Okutsu et al., 2006, Yoshizaki et al., 2011) who claim that SG shows at least a low level of migration in response to chemotactic stimulant. Except that, SG/OG has advantage than PGC on easy isolation from testes or ovary to ensure a large number of early stage germ cells for direct microinjection into the body cavity of recipient (Yoshizaki et al., 2011).

4.2. Isolation of germ cells

The effect of isolation is simplified by enzyme-induced dissolution of the germ cells from testicular tissue (Bellvé et al., 1977). Collagenase, trypsin and DNAse has been used for digestion process of sexually mature Nile tilapia, *Oreochromis niloticus* testis and isolated the SG from the two upper layer of a Percoll gradient (Lacerda et al., 2006).

Testes of adult male rainbow trout, *Oncorhynchus mykiss* were incubated in PBS (pH: 8.2) with 0.5 % trypsin and spermatogonia were isolated by flow cytometry (Okutsu et al., 2006). Hybrid recipient pearl danio (*Danio albolineatus*) larvae used as a sterile host and donor wild type zebrafish germ cells dissociated in 0.2% collagenase (Invitrogen) in PBS for 1 h at 28° C. After Percoll gradient purification, majority of eGC were obtained from 30%–35% and 25%–30% layers. 12 of 67 transplanted recipient pearl danio produced donor sperm (Wong et al., 2011). Pšenička et al (2015) used 2 to 4 years old Siberian

sturgeon (*Acipenser baerii*) as a donor. eGC isolation performed by enzymatic dissociation medium which composed of PBS with 0.2% trypsin and the testicular suspension separated by Percoll gradient. 10–33% layer of the gradient showed the highest percentage of eGC. Collected eGCs transplanted to the sterlet (*Acipenser ruthenus*) larvae (7 dph) by using microinjection. Identification of chimerism confirmed whit DEAD-family protein gene, DDX4 staining and 60% of host species showed proliferating donor PGCs (Pšenička et al., 2015).

GC dissociation and isolation technique for pikeperch which used in this study is based on these studies and optimized for practical use according to relevant data. I recommend using the 5–33% Percoll gradient for pikeperch GC isolation. I also recommend selection of early maturation stage males to obtain a higher concentration of GC compared to mature fish which produce high volume of spermatozoa and it causes decrease on early stage cell percentage.

For transplantation studies per larvae injection needed approximately 10 PGCs or 10,000 early germ cells (Okutsu et al., 2006, Takeuchi et al., 2003). According to my study results i can estimate 20.000.000 eGC can be isolated from one 18 month old pikeperch and 2000 larvae can be theoretically injected by these isolated eGCs.

5. Conclusion

Isolation of early stages of germ cells by enzymatic dissociation and Percoll gradient appears to be a practical and easy technique for further biotechnological studies in pikeperch. The effectiveness of the trypsin as a dissociation enzyme is species-specific as well as individually dependent and must be optimized for each fish species. This method is simple, it does not require complex laboratory equipment. This could be a feasible method for GC isolation in pikeperch. These data are also considered preliminary results for application in fish bioengineering by transplanting GC.

The early stages of germ cells also have other applications for biotechnology such as decreasing the required space for culture by using small fish as hosts, reducing the reproduction time by using host species which have shorter generation interval, detaining target species without keeping adult fish and obtaining knowledge for practice in other fish species.

Beyond that, cryopreservation of GC has great potential for maintaining and restoring threatened or commercially important fish species or lines. To achieve this, conservation of both paternal and maternal genetic information is essential. Although, cryopreservation technique has been successfully applied to the spermatozoa of many fish species, reliable methods are lacking for the long-term preservation of fish eggs and embryos. On the other hand PGC have potential to differentiate both male and female GC and this can help us overcome the obstacles about conservation of maternal genetic information.

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7. List of abbreviations

eGC	Early Germ Cell
GC	Germ Cell
GP	Germ Plasm
GCL	Germ Cell-less
GFP	Green Fluorescent Protein
GPCR	G Protein-coupled Receptor
HMGCR	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
IFITM1	Interferon Induced Transmembrane Protein
ISH	In Situ Hybridization
MDR49	Multidrug Resistance 49
OG	Oogonia
PGCs	Primordial Germ Cells
PKH26	Fluorescent Lipophilic Dye
RAS	Recirculation Aquaculture System
SG	Spermatogonia
SGPs	Somatic Gonad Precursors
SSCs	Spermatogonial Stem Cells
TEM	Transmission Electron Microscopy

8. Abstract in English

A practical technique for enzymatic dissociation and isolation of pikeperch (*Sander lucioperca*) (Percidae, Teleostei) early stage germ cells (eGC), including spermatogonia and spermatocytes, is reported in this study. Their potential to differentiate into functional gametes, and transmit genetic information to the next generations makes them suitable for cryopreservation and surrogate reproduction studies by germline chimera. Two different age groups (14 month old and 18 month old) of pikeperch were used to adjust the correct stage of eGC isolation. Finally the 18 month old samples were selected due to their high average gonadal volume (0.513 g). 10 ml PBS + 0.3% trypsin (304 mOsm/kg, pH 8) were used for enzymatic dissociation of testicular cells and they were sorted by centrifugation in Percoll density gradient. eGCs were identified on the basis of their ploidy level by CYSTAIN DNA 1 steps kit (PARTEC) and morphological characteristics trough by light microscopy. Cell counting was performed on histological sections and Percoll gradient layers whit the method of random square counting. The method of isolation enriched the number of eGC from 41.3% to 84.7%, obtained from the 33% of Percoll concentration.

Keywords: germ cells; isolation; Percoll gradient; flow cytometry; enzymatic dissociation; pikeperch

9. Abstract in Czech

Tato studie popisuje techniku enzymatické disociace a izolace raných stádií zárodečných buněk (eGC), zahrnující spermatogonie a spermatocyty candáta obecného (*Sander lucioperca*) (Percidae, Teleostei). Jejich potenciál diferencovat se do funkčních gamet a přenášet genetickou informaci do dalších generací z nich dělá vhodného kandidáta pro kryoprezervaci a tvorbu chimér. Pro zvolení vhodného věku pro izolaci byly testovány dvě skupiny candátů lišící se věkem (14 a 18 měsíců stáří). Díky vyšší průměrné hmotnosti gonád (0,513 g) se jako vhodnější ukázali 18 měsíční samci candáta obecného. Pro enzymatickou disociaci testes bylo použito 10 ml PBS s 0,3% trypsinu (304 mOsm/kg, pH 8). Testikulární buňky byly následně tříděny centrifugací v hmotnostním gradientu Percollu. eGCs byly identifikovány na základě jejich ploidní úrovně s použitím CYSTAIN DNA 1 kitu (PARTEC) a morfologických charakteristik s použitím světelné mikroskopie. Buňky byly počítány na histologických řezech a po použití Percoll gradientu metodou náhodných čtverců. Metoda izolace zvýšila zastoupení eGC ze 41,3% na 84,7% získané z 33% koncentrace Percollu.

Klíčová slova: zárodečné buňky, Percoll gradient, průtoková cytometrie, enzymatická disociace; candát obecný