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Embryo development and transplantation of primordial germ cells in pikeperch *Sander lucioperca*

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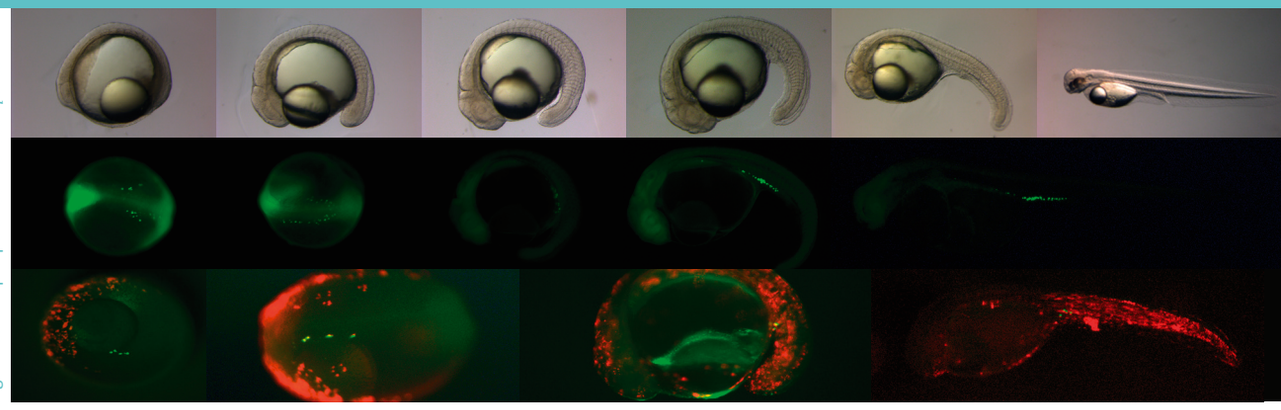

Hilal Güralp

Czech Republic, Vodňany, 2017

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Supervisor:
Assoc. Prof. Taiju Saito
University of South Bohemia in České Budějovice (USB) Faculty of Fisheries and Protection of Waters (FFPW) Research Institute of Fish Culture and Hydrobiology (RIFCH) Zátiší 728/II, 389 25 Vodňany, Czech Republic

Consultant:
Assoc. Prof. Martin Pšenička
University of South Bohemia in České Budějovice (USB) Faculty of Fisheries and Protection of Waters (FFPW) Research Institute of Fish Culture and Hydrobiology (RIFCH) Zátiší 728/II, 389 25 Vodňany, Czech Republic

Head of Laboratory of Reproductive Physiology:
Assoc. Prof. Martin Pšenička

Dean of Faculty of Fisheries and Protection of Waters:
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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Pikeperch as a model organism of Perciform

Sustainably feeding of the growing global population is a daunting challenge that is of interest to researchers, technical experts, and leaders the world over. Fish have a major role as a protein source. Aquaculture has grown at an impressive rate over recent decades, effectively meeting the increasing demand. The global fish production that is now supplied by aquaculture has reached 44% of the total (FAO, 2016). The technical procedures have been improved in supplying fish producing sustainably without using valuable natural resources and without destroying the aquatic environment. Now the breeding and other peripheral technologies have been well developed in many aquaculture species. Those techniques allow mass-production of fish under artificial condition. Furthermore, recent advances in the technology of reproductive biology have introduced new avenues of research in fish reproductive management for aquaculture. For example, chromosomal manipulation, embryonic manipulation and sex manipulation are available for production of sterilized fish and unisex population. While at the same time, it is important to maintain the natural biodiversity of fish species in aquaculture by integrating the genetic infrastructure and developmental biotechnology. For this aim, conservation and propagation of endangered and valuable genetic resources by a new reproductive technology which based on a living tissue transplantation so-called surrogate production has been developed for fish (Okutsu et al., 2007; Saito et al., 2008, 2011; Takeuchi et al., 2001; Yamaha et al., 2003). I explain more the benefits and methodology in the following text.

Pikeperch *Sander lucioperca* is a valuable recreational and commercial Percidae species in Europe, which is produced in polyculture with common carp or in recirculation systems (RAS) (Lappalainen et al., 2003; Dil, 2008; Philipsen, 2008; Zakęś and Demska-Zakęś et al., 2009). Pikeperch propagation has increased significantly in the last decade especially for intensive restocking programs in the leading countries of pikeperch production such as Denmark and Netherlands, in the meantime wild stocks have declined because of overfishing of marketable size pikeperch in central Europe (FAO, 2012; Steenfeldt et al., 2015). Sustainable aquaculture of pikeperch has been realized by natural spawning in ponds since the last century (Steenfeldt et al., 2015). Semi-natural spawning in tanks on artificial nests has progressed through the techniques of stimulating ovulation and spermiation with hormones such as human chorionic gonadotropin (hCG) (Donaldson, 1996; Křišťan et al., 2013). Today, producers prefer artificial fertilization of eggs which are then incubaated in Weiss-Zuger jars; egg stickiness is eliminated by treatment of proteolytic enzymes such as alcalase or tannic acid (Kristan et al., 2015; Żarski et al., 2015). These biotechnological developments provide opportunities to introduce new methodologies in the reproduction of pikeperch or its close relatives. Pikeperch, a member of Perciformes, has a potential to be a model fish species due to the relevant features such as ease of handling and breeding, and small space requirements and transparent, robust embryos that have been described in Chapter 2. Moreover, pikeperch has many marine fish relatives in Perciformes that make it more attractive in the case of surrogate production techniques that has been given more details about in following text of this chapter, Chapter 3 and 4.

Surrogate production techniques (surrogate propagation) is a method to produce target fish gametes through genetically different fish. Donor and host fish can be same or different fish species if donor germ cells successfully integrate and differentiate into gametes in host gonads. Surrogate production is an useful technique for the seedling production of aquaculture and the conservation of endangered fish species. There are different ways to produce surrogate fish. During pikeperch development, the embryonic period is the most available to be able to perform surgical manipulations for germ cell transplantation since

embryos can be kept under the artificially controlled environment. Therefore, I focused on the methods, blastomere and blastoderm transplantation, to produce germline chimeras in pikeperch. It is easy to perform and provide an advantage to close relatives between donor and host that have been given more details about in following the text of this chapter and chapter 3.

The importance of embryo development studies

Knowledge of embryo development is essential for developing new applications such as micromanipulation techniques, especially the transplantation of germ cells from the embryo of one species into another species to produce chimeras (Nakagawa et al., 2002; Saito et al., 2008; Takeuchi, 2003). During normal embryo development, mortality or occurrence of abnormalities caused by the intrinsic/extrinsic factors on embryogenesis is the inevitable. A critical point of experimental manipulation can be distinguished by defined morphological characterization of stages in the donor or host embryos, so that development failures caused by manipulation can be easily detected. The current knowledge on embryo development has been defined in zebrafish *Danio rerio* (Kimmel et al., 1995), loach *Misgurnus anguillicaudatus* (Fujimoto et al., 2006), summer flounder *Paralichthys dentatus* (Martinez, et al., 2003), ice goby *Leucopsarion petersii* (Arakawa et al., 1999), medaka *Oryzias latipes* (Iwamatsu, 2004), goldfish *Carassius auratus* (Tsai et al., 2013), the North American percids fantail *Etheostoma flabellare*, rainbow darter *Etheostoma caerulein*, banded darter *Etheostoma zonale*, logperch *Percina caprodes*, yellow perch *Perca flavescens*, and walleye *Stizostedion vitreum*, as well as the European species ruffe *Gymnocephalus cernuus*, Balon's ruffe *Gymnocephalus baloni*, yellow pope *Gymnocephalus schraetser*, and Danube streber *Zingel streber* (Cooper, 1978; Kovac, 1994, 2000; Mansueti, 1964; McElman et al., 1979; Paine et al., 1984a; Paine et al., 1984b). However, duration and timing of embryonic developmental stages are not identical among all species, although there are similarities associated with egg features and developmental specificities such as cleavage pattern (Figure 1). To date, temperature effect on developmental stages of pikeperch embryos have not been well-described (Lappalainen et al., 2003; Oprea et al., 2014; Schlumberger et al., 1996; Zakęś and Demska-Zakęś, 2009). Documentation of embryo development characteristics was required to be able to obtain more information such as about germ cell development to implement germ cell transplantation. During embryo development, the fate of every cell build up the organism after a specific moment (Blaser, 2005; Kane and Kimmel, 1993; Lee et al., 2014; Urven et al., 2006). Therefore, we must observe germ cell development in each species rather than simply comparing embryos to estimate the fate of the germ cells. In the following text, we provide detailed information on the techniques for observation the germ cell formation and development mechanism. We compare several methods, including the one that we used for tracking germ cells (Chapter 4). To be able to apply germ cell transplantation technique to pikeperch, it is necessary to have more information about developmental limitations such as temperature effect on the developmental rate – see details in Chapter 2.

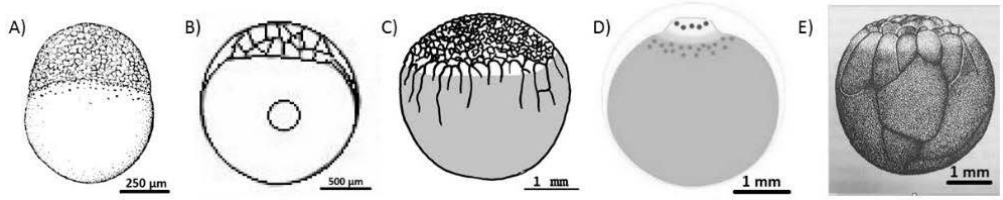

Figure 1. *Various meroblastic (A–D) and holoblastic (E) cleavage patterns and yolk structures during blastula stage in A) zebrafish (Kimmel et al., 1995), B) summer flounder embryo has oil globule within yolk (Martinez and Bolker, 2003), C) longnose gar (Long et al., 2001), D) Atlantic salmon embryo has big proportion of yolk (Nagasawa et al., 2013), E) sturgeon (Dettlaff et al., 1993)*

Germline stem cells in fish

Germline stem cells (GSCs) represent primordial germ cells (PGCs) in an embryo or spermatogonial/oogonial stem cells in larvae which are precursors of subsequent gametes in fish. In recent years, much has been learned about the origin of germ cells in fish, the germ cells originate at a species-specific area in the embryo and migrate to the developing gonadal ridges during embryo development (Li et al., 2015; Linhartova et al., 2014; Presslauer et al., 2012; Saito et al., 2006; Saito et al., 2014). There, they undergo gametogenesis ending with cellular differentiation into mature gametes, either eggs or sperm.

Germplasm and germ cell formation

PGC development was initially identified by light and electron microscopy for several species of fish such as zebrafish, medaka, goldfish, guppy, and trout (Bruslé and Bruslé, 1978; Eddy, 1976; Johnston, 1951; Satoh, 1974). PGCs have round-large nuclei, possess prominent nucleoli, distinct nuclear membrane and a large cytoplasm in comparison with other cell types (Johnston, 1951). Johnston (1951) observed PGCs in the posterior hypoblast during gastrulation in largemouth bass (Figure 2A–B). Gamo (1961) identified PGCs during early and mid-gastrula stages within ectoderm and mesoderm and during neurula stages within endoderm in medaka. The results obtained with electron microscopy aim to reveal mostly germplasm. Germplasm-like structures or germplasm, which are described within dense particles, mitochondrial clusters chromatoid or electron-opaque substances are found in positions where putative PGCs are formed in several teleosts such as medaka, mullet and carp (Bruslé and Bruslé, 1978; Eddy, 1976; Hamaguchi, 1982; Hogan, 1978; Satoh, 1974).

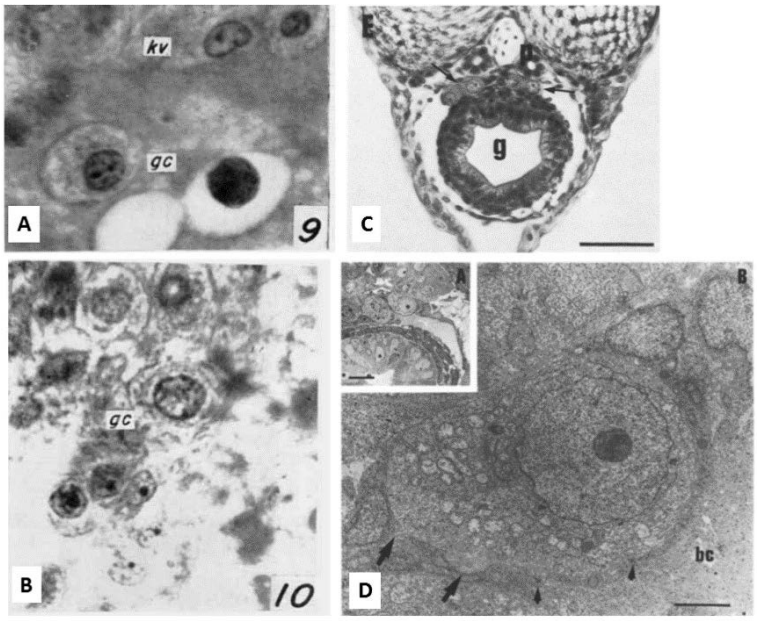

Figure 2. *The histological features and position of primordial germ cells with their large size, definite nuclear and cellular membranes, their darkly staining nuclei and hyaline cytoplasm, and the extrusion of plasmosomal to migrate independently, (A–B) the largemouth black bass, A) A portion of the periblast ventral in a 22-hour the largemouth black bass embryo, gc: germ cell, kv: vesicle, x 1500. B) An enlargement of the germ cells and periblast what appears to be pseudopodial extensions of the cytoplasm, x 1500 (adapted from Johnston, 1951) (C–D) medaka, C) All germ cells are in the gonadal anlage between the pronephric duct and the gut at stage 30. Scale 40 µm, D) a) Two PGCs have reached the gonadal region, and one is in the somatic mesoderm. Scale 10 µm. b) High magnification of the PGC in the somatic mesoderm. The cytoplasmic sheet of mesodermal cells completely wraps around the PGC. Small cytoplasmic protrusions of the PGC are shown by large arrows. Scale 2 µm; bc: body cavity (adapted from Hamaguchi, 1982).*

Germplasm maternally provided and contains mitochondrial clusters, RNA, and proteins which are needed for PGC formation, migration, and development (Hashimoto et al., 2004; Herpin et al., 2007; Yoon et al., 1997). After identification of molecular markers of germplasm such as *vasa (vas), dead end (dnd), nanos (nos), bucky ball (buc), dazl (daz)* genes, germplasm formation and localization could be described. In zebrafish, the germplasm is formed from two classes of germplasm components.The first class is RNAs which are initially present in the animal pole of mature oocytes, such as *dnd, nanos3* and *vasa* RNA, whereas the second class of germplasm components includes *daz1* vegetally localized during oogenesis through messenger transport organizer pathway recruited to the germ plasm during early cleavage (Theusch et al., 2006). In sturgeon, the germplasm observed at vegetal pole of the embryo by labeling technique which I described in following text in detail (Saito et al., 2014). In teleost, genetic analysis of endogenous patterns of RNA localization of *dnd, nos1, vas, buc* and *dazl* RNAs during the first cell cycle have shown germplasm forms at the furrows of the first and second cleavage divisions where putative PGCs are formed (Bontems et al., 2009; Hashimoto et al., 2004; Herpin et al., 2007; Li et al., 2015; Nagasawa et al., 2013; Presslauer et al., 2012; Theusch et al., 2006; Wang et al., 2015). The initial germplasm assembly is actin-dependent, whereas the subsequent segregation requires the function of the so-called furrow

microtubule array (FMA) (Knaut et al., 2000; Theusch et al., 2006). The inhibition of FMA remodeling has shown myosin activity is required for the distal aggregation of the zebrafish germplasm (Urven et al., 2006). The germplasm concentrates into four subcellular clumps by the 32-cell stage that is segregated to four cells through the 1k cell stage in zebrafish (Figure 3) (Knaut et al., 2000).

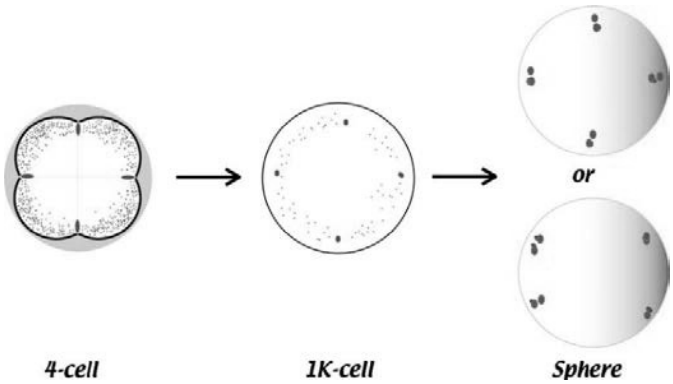

Figure 3. *Illustration germplasm containing vasa mRNA is enriched in the vicinity of the cleavage furrows (blue stripes and small blue dots) from 4-cell stage to sphere in zebrafish embryo of view from the animal pole (adapted from Raz, 2002).*

Germ cell migration and survival

In animal development, during blastula stages, zygotic transcription is activated in PGCs and soma, and cells begin to migrate to the region to construct particular tissue (Blaser, 2005; Kane and Kimmel, 1993; Lee et al., 2014; Newport and Kirscner, 1982; Urven et al., 2006). In teleost, this period has been described as a transition from synchronous to asynchronous cell cycle known as midblastula transition (MBT) (Figure 4) (Fujimoto et al., 2004; Iwamatsu, 2004; Kane and Kimmel, 1993; Yamaha et al., 1999). There are ten synchronous cell cycles in developing, loach and medaka embryos (Fujimoto et al., 2004; Iwamatsu, 2004; Kane and Kimmel, 1993) whereas goldfish have 9 synchronous cell cycles (Yamaha et al., 1999) and zebrafish have 8 synchronous cycles continues during 9^{th} cycle (Kane and Kimmel, 1993). After these synchronous divisions are completed, the length of S phase increases, G1, and G2 phases intervene between M and S, the cell cycle rate is slow, and synchrony is lost (Kane and Kimmel, 1993). Understanding of timing of asynchronous cell division helps to estimate the time of the MBT and onset of PGCs migration and division in pikeperch (Chapter 3).

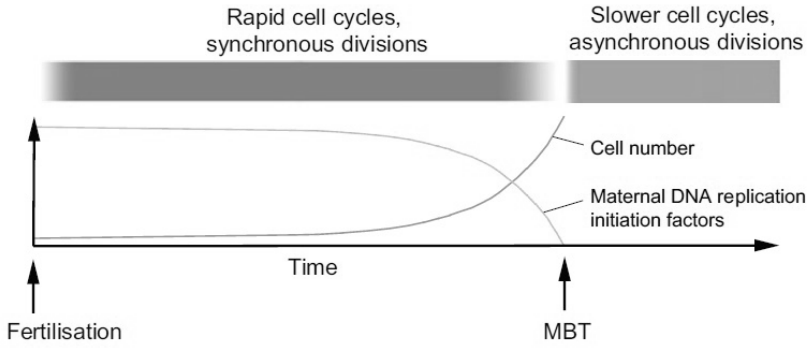

Figure 4. *Cell cycle changes during the maternal to zygotic transition. Synchronous cell divisions switch to asynchronous cell divisions. Maternal DNA replication decrease which leads a slow S phase at the MBT. Cell cycle length increases within gap phases (adapted from Langley et al., 2014).*

In several fish, the migration pattern of PGCs is varied and species-specifically associated with egg features and developmental specificities such as cleavage pattern and MBT (Herpin et al., 2007; Linhartova et al., 2014; Saito et al., 2006; Saito et al., 2014; Wang et al., 2015). Several reports have shown the PGC migration of fish species, including zebrafish, pearl danio, goldfish, loach, herring, medaka, ice-goby, tench, cod, sturgeon, olive flounder (Li et al., 2015; Linhartova et al., 2014; Presslauer et al., 2012; Saito et al., 2006; Wang et al., 2015). All these reports are based on two main sophisticated methods for tracking migrating PGCs which are *in situ* hybridization (ISH) and green fluorescent protein (GFP) labeling techniques by injecting mRNA designed for that purpose. The ISH provides an advantage of tracking PGCs by the target transcripts of the probe which can be labeled by complementary DNA/RNA on a slice of fixed tissue or a whole embryo from the beginning of the cleavage (Jin and Lloyd, 1997). On the other hand, the PGCs can be visualized after the beginning mid-gastrula stage of embryogenesis by injecting synthetic mRNA constructed with a GFP sequence into 1–2 cell stage embryos (Köprunner et al., 2001; Saito et al., 2006; Yoshizaki, 2005). Saito et al. (2006) showed that zebrafish *nos3* 3′ untranslated region (UTR) mRNA was conserved among other teleost species and could label PGCs of many fish species. This GFP labeling technique using zebrafish GFP-*nos3* mRNA was performed in pikeperch (Chapter 4).

There are more genes relevant to PGC migration and development, and some of them have been already identified as responsible genes for germplasm assembly either PGC formation (Table 1). Weidinger et al. (1999) have shown that the *vasa* mRNA, DEAD box RNA helicase, is a reliable and stable marker for tracing PGCs during the development of zebrafish. Köprunner et al. (2001) have also demonstrated that *nos3* mRNA, RNA-binding zinc finger proteins, is incorporated into the PGCs during blastula stages and forms two bilateral clusters close to the position of the future gonad during the first day of development of zebrafish embryos. Another gene responsible for migration is dnd gene. The function of dnd gene is investigated by morpholino gene knockdown, which is commonly used in fish gem cell biology studies (Ciruna et al., 2002; Linhartová et al., 2015; Slanchev et al., 2005; Wargelius et al., 2016; Weidinger et al., 2003). As a different vertebrate germ plasm component, *dnd* RNA-binding protein is essential for pseudopodia formation in PGCs by implication for PGCs migration (Liu et al., 2009; Nagasawa et al., 2013; Wang et al., 2015; Weidingeret al., 2003).

The mechanism of PGC migration is well conserved among animals from fruit fly to human (reviewed by Richardson and Lehmann, 2010). PGC migration is regulated by multiple factors,

including the molecules above (e.g. *nanos, dnd*). However, a combination of attractant stromal-derived factor (*sdf1a*), G-protein Chemokine receptors as repellent *cxcr7b* and receiver *cxcr4b*, is called chemokine signaling, orients PGCs to the directions towards the gonadal ridge (Figure 5) (Kunwar and Lehmann, 2003; Richardson and Lehmann, 2010). PGCs encoded by *cxcr4b* follow the signs of *sdf1a* which is produced by the somatic cells in the way of migration (Kunwar and Lehmann, 2003). The mechanism of PGC migration has been investigated in zebrafish and medaka (Braat et al., 1999; Doutsidou et al., 2002; Knaut et al., 2003; Raz, 2002; Weidinger et al., 2002; Yoon et al., 1997; Yoshizaki et al., 2002).

Table 1. *Gem cell gene activity/function (summarized from Li et al., 2012; Xu et al., 2010).*

Gene	Description	Function			
		Germplasm Assembling	**PGC Formation**	**PGC Migration**	**PGC Survive**
vasa	DEAD box helicase	✓	✓	✓	
ziwi	RNA interacting proteins			✓	✓
bucky ball	Novel zebrafish gene	✓	✓		
nanos1 to 3	RNA-binding zing finger proteins	✓	✓	✓	✓
staufen	RNA-binding proteins			✓	
dead end	RNA-binding proteins			✓	✓
Hmgcr	3-hydroxy-3-methylglutaryl coA reductase			✓	
sdf1a-cxcr4b	Stromal-derived factor 1a/ Receptor			✓	

The genetic analysis of the mechanism of PGC migration in zebrafish has shown that inactivation of encoding of *cxcr4b* proteins by morpholino antisense knockdown has caused failure of PGCs to reach the gonadal ridge and instead migrate to different and irrelevant places in the fish (Doutsidou et al., 2002; Knaut et al., 2003). Although these chemokine receptors guides PGCs and various cell types such as mesendodermal or neural cells for migration, each cell type is able to migrate on their pathways by using the chemokine receptors and there is more to understand the details of the chemokine signalling mechanism of PGC migration (Xiang et al., 2002; Zou et al., 1998).

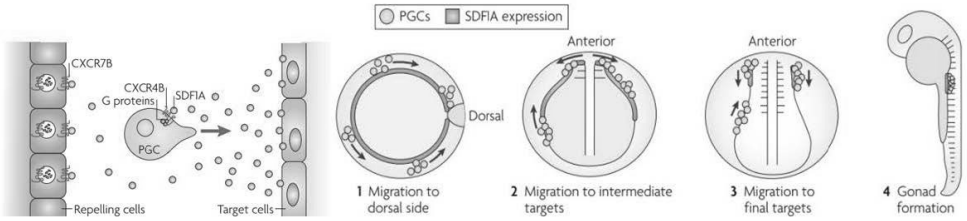

Figure 5. *Illustration of chemokine signaling mechanism and pathway of PGC migration in zebrafish (adapted from Richardson and Lehmann, 2010).*

There is also an enzymatic pathway in PGC migration which is described in fruit flies and in zebrafish. 3-hydroxy-3-methylglutaryl coenzyme reductase (HMGCR) also known as Columbus expresses in the lateral mesoderm with a role in the production of a PGC attractant (Thorpe et al., 2004). Although the mechanism how PGCs complete their migration is still unknown, it seems that the duration of migratory activity of PGCs is controlled autonomously (Saito et al., 2010).

In chapter 4, we visualized PGCs by zebrafish GFP-*nos3* mRNA and observed their migration in pikeperch embryos.

Surrogate production in fish

In mammals, surrogate production means that a host carries and delivers the zygote from a donor(s). In other animals, for example in chickens, this kind of production is established by transferring PGCs to the blood stream of developing chicken embryos (Tajima et al., 1993). Transplanted donor PGCs are able to incorporate into the host gonad, and produces donor-derived offspring.

Surrogate production in fish is established as a technique to obtain the gametes of a certain genotype through the gonad of another genotype by transplanting germline stem cells during early development (Takeuchi et al., 2001; Yamaha et al., 2003; Okutsu et al., 2007; Saito et al. 2008, 2011). A key factor for implementation of surrogate production in fish is how to incorporate donor germ cells into the host gonad. If the transplanted germ cells survive, localize in the gonadal ridge and give rise functional gametes, the host is called a germ-line chimera and the gametes of a donor fish can be obtained via the chimera (Yamaha et al., 2007).

Production of endangered or commercially valuable fish can benefit from germ cell transplantation by reducing the time for fish to reach sexual maturity and the space required for aquaculture (Pšenička et al., 2015), banking the genetic resource by cryopreservation of germ cells (Linhartova et al., 2014; Pšenička et al., 2016), producing fish by *in vitro* cultivation of germ cells without maintaining broodfish (Shikina et al., 2013), and the possibility of producing marine fish inland using freshwater species as a host (Saito et al., 2011).

To date, the production of germline chimeras is performed with several additional methods such as visualization, isolation, identification of germ cells, sterilization of host and transplantation (Okutsu et al., 2006a; Saito et al., 2011; Linhartová et al., 2015). Moreover, generation of germline chimera could be permanent when cryopreservation and *in vitro* cultivation of germ cells are combined (Morita et al., 2015). The experiments carried out up to now for these purposes are described below.

Micromanipulation methods

PGC transplantation

Among the varied approaches to generate germline chimera in teleosts, is blastomere transplantation, applied in zebrafish, medaka, trout and loach (Lin et al., 1992; Nakagawa et al., 2002; Saito et al., 2011; Takeuchi et al., 2001; Wakamatsu et al., 1993; Yamaha et al., 2001, 2003). Firstly, Lin et al. (1992) described how blastomeres were randomly aspirated into a micro-glass needle from a donor embryo and transplanted into a host embryo at the same stage. In this study, they used wild-type striped donor embryos of zebrafish and albino zebrafish host embryos. They confirmed germline chimerism by the morphology of fish and

molecular analysis. Investigation of the PGCs localization in teleost embryo was only the initial knowledge which was required for germ cell transplantation (Yoon et al., 1997). In teleost, the PGCs localize around the peripheral region of the blastoderm at blastula (Yoon et al., 1997), and it is hard to distinguish the PGCs from the somatic blastomeres. Therefore, Yamaha et al. (2001) transplanted the PGC-containing lower layer of blastoderm of donor triploid crucian carp between two half of the blastoderm of a host diploid goldfish embryo. A key factor for implementation of the surrogate production in fish is how to incorporate donor germ cells into the host gonad. Yamaha and his colleagues (2001) showed that their technique improved the efficiency of germline chimera production. In chapter 3, therefore, I applied the "blastodisc sandwiching" technique for inducing germline chimeras in pikeperch, and also for testing the optimal timing of surgical manipulations on embryos of this species.

Some researchers have used the blastomere transplantation technique to investigate the function of maternal RNAs (Ciruna et al., 2002; Giraldez et al., 2005). Through micromanipulation, PGCs were harvested from donor embryos and transplanted into blastula-stage recipients that had endogenous PGC development blocked by the injection of a *dead end* antigen morpholino. The germline chimerism after blastomere transplantation was confirmed by observation of labeled PGCs as shown by Saito et al. (2006).

The limitation of this procedure is the nature of the fish embryo, such as the feasibility of dechorionation under artificial conditions. Also, the blastomeres transplantation technique is limited by the phylogenetic relationship distance between a donor and a host. Somatic blastomeres have pluripotency, i.e. they can differentiate into any cell types except PGCs until gastrula. The transplanted blastomeres are able of incorporate to the development of recipient without discrepancy of tissue that can cause deformation of the embryo. Also, donor-derived PGCs can efficiently migrate toward the gonadal ridge of a host embryo after blastomere transplantation (Kawakami et al., 2010; Saito et al., 2008; Takeuchi et al., 2003). However, blastomere transplantation including PGCs can be effected between same or closely related species. Otherwise, somatic cells can disturb the embryo development through induction of abnormality and inhibition of PGCs migration (Saito et al., 2010).

To generate germline chimera between distantly related species without malformation in the chimeras, the PGCs are isolated from donor embryos after labeling with GFP-*nos3* 3'UTR mRNA, which is the technique that was mentioned above (Köprunner et al., 2001, Saito et al., 2006). GFP-labeled donor zebrafish PGCs can be isolated from the embryos by using enzyme treatment and aspirating into a micro-glass needle, then transplanting them into sterilized host pearl danio embryos (Saito et al., 2008). The identification of chimerism is analyzed by *vasa* expression and genotyping. It is reported that 50% donor-derived PGCs migrated to the gonadal ridge of pearl danio (Saito et al., 2008). Saito at al. (2010) reported that the 10–15 somite stage of donor zebrafish embryo was optimal for isolating PGCs for efficient somatic cell-free PGC transplantation into pearl danio, goldfish, and loach. Moreover, 45% donor-derived single PGC successfully migrated to the gonadal ridge of the host as different species. Experiments on more distantly related species such as between Japanese eel and zebrafish or sturgeon and goldfish confirmed that transplantation of single purified PGC will produce xenogeneic chimeras (Saito et al. 2011, 2014). However, this method has drawbacks such as the existence of the limited amount of PGCs in donor and requirement of additional steps before performing transplantation.

PGC transplantation has a different dimension since performing in hatched embryos as a host (Takeuchi et al., 2003, 2004; Yoshizaki et al., 2005). 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. They reported that GFP-labeled donor-derived PGCs could incorporate into the host gonad after intraperitoneal

cavity injection when it is performed at the same or younger stage of hatched host embryo in salmonids, and giving rise to female or male germ cells in the chimeras. The xenogeneic chimeras produced by this technique produced donor-species derived offspring. Lack of an active immune system in the host embryos at transplantation prevents immune rejection of donor-derived germ cells. PGC transplantation into the newly hatched embryos was reported to be relatively less complicated than single PGCs transplantation reported by Saito et al. (2008) but, even so, the success of chimerism is comparatively lower.

Spermatogonia and oogonia (early-stage germ cell) transplantation

Spermatogonia transplantation has been previously applied to mammals (Brinster and Zimmermann, 1994). It was successfully developed in fish using the microinjection technique developed on hatched embryos as shown above (Okutsu et al. 2006b, 2007, 2008; Majhi et al. 2009, 2014; Nóbrega et al., 2010; Lacerda et al., 2010). The technique covers the spermatogonial stem cells isolation by enzyme treatment from a gonad of a donor fish and the transplantation of these cells into the intraperitoneal cavity of a recipient. The main advantage of this method is that a large number of germ cells can be obtained from donor testes or ovary.

Okutsu et al. (2006b) described the procedure from isolation and collection of testicular germ cells containing spermatogonial stem cells of donor adult male rainbow trout to transplantation into the peritoneal cavity of newly hatched embryos of both sexes. They used transgenic trout as a donor in which testicular spermatogonia were labeled by the GFP-*vasa* so as to microscopically track in the host. They dissociated transgenic donor testis by enzyme treatment (0.5% trypsin) and isolated testicular germ cells from other cell types and fat by microfiltration. The identification of chimerism was analyzed by cell sorting with flow cytometry, PCR and progeny testing. Transplanted adult testicular germ cells were incorporated into sexually undifferentiated embryonic gonads and undergo gametogenesis. The donor-derived germ cells differentiated into spermatozoa in male recipients and fully functional eggs in female recipients. These results illustrated the plasticity and bipotentiality of spermatogonial cells (early-stage germ cells) in fish (Nóbrega et al., 2010). Sunsequently, Okutsu et al. (2007) produced germline chimeras by transplanting rainbow trout spermatogonia from adult testis into sterile triploid hatched larvae of masu salmon, and they showed that the germline chimeras gave rise to only gametes of the donor. Transplanted spermatogonia colonized and developed in recipient testes and formed fertile sperm with the donor genotypic characteristics.

Many variations of this technique have been applied in several species with different donor cell type, labeling methods, host sterilization techniques, and host stages (Higuchi et al., 2011; Lacerda et al., 2006; Majhi et al., 2009, 2014; Morita et al., 2012, 2015; Psenicka et al., 2015; Wong et al., 2011; Yazawa et al., 2013). For example, Lacerda et al. (2006, 2010) performed spermatogonia transplantation in Nile tilapia strains in which spermatogonia were transplanted through the urogenital papilla of chemically sterilized adult fish. They used Percoll gradient method to sort out testicular cells and labeled them with the fluorescent lipophilic dye (PKH26-GL) and trypan blue. Their results indicated that spermatogonial transplantation could be applied successfully in sexually mature host fish. The analysis of testis of host individuals demonstrated PKH26-labeled germ cells in the lumen of the seminiferous tubules and reported that there were no immune rejections in the adult allogeneic recipient. Majhi et al. (2009) performed the same technique with CFDA-SE-labeled testicular cells, and the chimeras generated donor-derived sperm within six months after transplantation. Wong et al. (2011) produced germline chimeras by transplanting ovarian germ cells into sterile zebrafish hybrid recipients. They isolated ovarian germ cells from 3 months old adult Tg (*vasa:* DsRed2-

vasa); Tg (*bactin:* EGFP) double transgenic zebrafish after enzyme treatment and Percoll gradient. Their result showed that transplanted ovarian cells colonized in the host gonads and produced functional sperm. Pšenička et al. (2015) reported that the early-stage germ cell (mainly spermatogonia) isolation and transplantation technique could be performed in Siberian sturgeon with 60% success of proliferation of donor-derived female and male germ cells in recipients.

On the other hand, some studies have reported that germline stem cells from the zebrafish or trout testis or ovary could be cultured under the artificial condition, and even these cells can transmit the germ-line after transplantation (Sakai et al., 2002; Shikina et al., 2008). This promising implementation in biotechnology could be applied to preservation of the genetic stock of valuable animals or endangered species.

Host sterilization methods

Sterilization host individuals is required to be able to generate a germ-line chimera which exclusively produces the donor-derived functional gametes. There are several ways to sterilize host fish such as a gene knockdown with morpholino oligos, gynogenetic applications, and chemical treatments.

The gene knockdown with morpholino oligos is based on inhibition of the function of genes which are responsible for germ cell formation, migration, and development. Some of the germ cell transplantation studies such as Saito et al. (2008) and Linhartová et al. (2015) used anti-*dead end* (*dnd*) morpholino oligonucleotides in zebrafish and sturgeon, respectively.

Sterilization with chemical agents such as busulfan (1,4-butanediol di-methanesulfonate) is used mostly for adult recipients for germline chimera production purpose like in Lacerda et al. (2006, 2013) studies. Earlier, busulfan was used for cancer treatment because of its effect on uncontrolled dividing cells (Brinster, 2002).

Hybridization is also one method to obtain infertile fish. Hybrids can be sterile in the case of crossing distantly related parental species that have chromosome pairing complications during meiosis (Piferrer et al., 2009). Yamaha et al. (2003) used a hybrid of sterile goldfish × common carp as a host and fertile goldfish embryos as a donor in the purpose of chimeric fish production. To obtain sterile goldfish × common carp hybrid males, all female XX goldfish and YY super-male carp were crossed. Some hybrids grow faster than original species of parents, and it is another advantage. Wong et al. (2011) used a female zebrafish x male pearl danio hybrid as a sterile host for ovarian cells transplantation.

Triploidization is another way to obtain infertile individuals in fish. Triploid organisms have three sets of homologous chromosomes; some are found spontaneously in nature but it is possible to produce triploids in many commercially related species of fish (Legatt and Iwama, 2003; Maxime, 2008).There are several mechanisms to induce triploidy, such as hydrostatic pressure, temperature or chemical shock to eggs after fertilization so as to inhibit the extrusion of the 2nd maternal meiotic polar body; the haploid set of maternal chromosomes provides the third set (Malison et al., 2001; Dunham, 2004). Another way to obtain triploid fish is crossing diploid and tetraploid fish (Francescon et al., 2004; Nam and Kim, 2004). Okutsu et al. (2007) used triploidization in the purpose of sterilization of host masu salmon embryos for germline chimera production. There is a recent report on efficient pikeperch triploidization by heat shock (Blecha et al., 2016). If the tryploid pikeperch is the sterile, they could be used as a host for germ cells transplantation technique.

Objectives of the thesis

The current study was devoted to the comprehensive description of the development of embryos related to germ cell transplantation in pikeperch and pursuing the following objectives:

1. To document stages of pikeperch embryo development to first feeding time and demonstrated effects of different temperatures on the rate of embryogenesis to determine the temperature limits for reducing the speed of development with minimum negative effect on growth, and survival rate.
2. To describe the important embryonic events that are called yolk syncytial layer (YSL) formation and midblastula transition (MBT) during the blastula stage in pikeperch to obtain basic information about the timing of a micromanipulation technique.
3. To use GFP labeling to describe the migration of PGCs from gastrula through hatching, and to evaluate the potential of blastomere transplantation in pikeperch.

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CHAPTER 2

DEVELOPMENT, AND EFFECT OF WATER TEMPERATURE ON DEVELOPMENT RATE, OF PIKEPERCH *SANDER LUCIOPERCA* EMBRYOS

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DEVELOPMENT, AND EFFECT OF WATER TEMPERATURE ON DEVELOPMENT RATE, OF PIKEPERCH *SANDER LUCIOPERCA* EMBRYOS

H. Güralp*, K. Pocherniaieva, M. Blecha, T. Policar, M. Pšeničkа, T. Saito

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic
**Corresponding author.*

ABSTRACT

Knowledge of embryo development is essential to the application of reproductive biotechnology in aquaculture, including for pikeperch *Sander lucioperca*. We describe pikeperch embryo development and demonstrated effects of temperature on the duration of embryogenesis. Developmental stages in embryos incubated at 15 °C were identified as zygote, 0–1.5 hours post-fertilization (hpf); cleavage, 2.5–7.5 hpf; blastula, 9–18.75 hpf; gastrula, 21–39, hpf; segmentation, 45–105 hpf; and hatching, 125–197 hpf. Additional groups of eggs were fertilized and incubated at 10, 15, 20, and 25 °C to document stages of development, development rate, and survival. The optimal fertilization and incubation temperature was shown to be 15 °C, with the highest fertilization, survival, and hatching rates. Embryo development was slower at 10 °C, with 45% of fertilized embryos surviving to hatching. Development was accelerated at 20 °C, and resulted in a 56% survival rate of fertilized embryos. At 25 °C, embryos did not develop to the blastula stage. Pikeperch could be a valuable percid model for research in which flexible incubation temperatures is required.

Keywords: *embryogenesis, insemination, incubation, pikeperch, temperature tolerance*

Introduction

Fish embryo development is a critical aspect of developmental biology, and knowledge of its characteristics is essential to improving reproduction techniques for commercially valuable and endangered species. The developmental stages of many species have been described in detail. Currently, zebrafish *Danio rerio* (Kimmel et al., 1995), medaka *Oryzias latipes* (Iwamatsu et al. 1994, 2004), ice goby *Leucopsarion petersii* (Arakawa et al., 1999), and goldfish *Carassius auratus* (Tsai et al., 2013) are the preferred model fish species in studies of developmental biology. Developmental stages have also been described in summer flounder *Paralichthys dentatus* (Martinez et al., 2003), Pangas catfish *Pangasius pangasius* (Ferosekhan et al., 2015), and striped snakehead *Channa striatus* (Marimuthu et al., 2007), which are cultured species with increasing demand. Commercially valuable, as well as endangered, species including tench *Tinca tinca* (Linhartova et al., 2014), loach *Misgurnus anguillicaudatus* (Fujimoto et al. 2004, 2006; Zhao, 2012), and Siberian sturgeon *Acipenser baerii* (Park et al., 2013) are attracting interest with respect to advanced biotechnologies such as generation of chimeric fish and clonal production (Nakagawa et al., 2002; Pšeničkа et al., 2014).

Pikeperch *Sander lucioperca* is a freshwater perciform teleost highly valued in aquaculture. In Western Europe, pressure for the conservation of natural pikeperch stock has led to its intensive production in recirculating aquaculture systems (RAS) (Zakęś et al., 2016). To improve broodstock management in RAS, whether for scientific investigation or commercial hatchery

production, studies have focused primarily on egg and larva quality (Hermelink et al., 2011; Křišťan et al., 2013; Németh et al., 2012; Zarski et al., 2013). Pikeperch reproductive features, including production of large numbers of eggs (200000 kg^{-1} body weight) and accessible, transparent, robust embryos; a relatively short incubation period [65–110 degree days (dº)] (Schlumberger et al., 1996; Zakęś et al., 2016) and its tolerance to micromanipulation, makes pikeperch a good potential candidate as a surrogate host or donor of perciforms (Güralp et al., 2016). However, micromanipulation is successful for only a limited time during a specific period in embryogenesis (Güralp et al., 2016; Saito et al., 2011; Yamaha et al., 2003). Knowledge of the developmental rate of pikeperch embryos relative to water temperature may allow retardation of development for more successful micromanipulation.

Temperature is among the most influential of environmental factors on development of percid embryos (Feiner et al., 2015), especially during fertilization and incubation of embryos, and is species-specific (Cooper, 1978; Kováč, 1994; Kovác, 2000; Mansueti, 1964; McElman and Balon 1979; Paine and Balon, 1984a,b). Knowledge of the temperature range at which normal development occurs can limit mortality related to temperature fluctuations that exceed safe limits. The optimal temperatures for pikeperch spawning (8–16 °C), fertilization (12–16 °C), and incubation (11.5–20 °C) are reported to be among the highest in investigated percids (Schlumberger et al., 1996; Feiner et al., 2015). Oprea et al. (2014) studied effects of two temperature ranges on the duration of pikeperch embryogenesis in eggs incubated under hatchery conditions. Duration of development was 9 days at 9–13 °C and 6 days at 13–16 °C with high rates of fertilization, survival and hatching (Oprea et al., 2014).

Development of embryos has been documented in several percids (Mansueti, 1964), but development stages of pikeperch embryos are not. The goal of this study was to characterize the stages of pikeperch embryo development to first feeding at 15 °C, to investigate effects of temperature on development rate, and to determine incubation temperatures that retard development while minimizing negative effects on survival and growth.

Material and methods

Ethics

All experimental procedures followed the principles of laboratory animal care and national law 246/1992 Animal Welfare on the protection of animals, and the criteria of the EU Harmonized Animal Welfare Act of the Czech Republic.

Fish source

Three female and three male *Sander lucioperca* broodfish were maintained in an RAS at the University of South Bohemia, Faculty of Fisheries and Protection of Waters, during the spawning season from March to April 2016. The fish were anesthetized with an aqueous solution of 30 mg L^{-1} clove oil (Dr Kulich Pharma, s. r. o., Czech Republic). Human chorionic gonadotropin (Chorulon) was injected intramuscularly at 500 IU kg^{-1} body weight to induce ovulation and spermiation (Blecha et al., 2016). Eggs and sperm were collected at ~87 dº post-injection.

Preparation of embryos for characterization of embryonic stages at 15 °C

Eggs were fertilized in carbon-filtered and aerated tap water at 15 °C. A group of embryos was prepared for monitoring, photographing, and documenting development by removing the chorion.

To remove the chorion, two minutes post-activation, embryos were treated with 0.2% trypsin and 0.4% urea in Ringer´s solution buffered by 10 mM TAPS to pH 8.5. After 10 minutes embryos were transferred into 1.6% albumen in Ringer´s solution buffered with 10 mM HEPES to pH 7.5 to stop the enzymatic reaction. The stickiness was removed, and the softened chorion was removed using fine forceps as previously described due to the low surface tension of embryos (Güralp et al., 2016). The dechorionated embryos were divided into five groups of 50, placed in 1% agar-coated Petri dishes, and incubated in 1.6% albumen in Ringer´s solution buffered with 10 mM HEPES to pH 7.5 until somitogenesis (Güralp et al., 2016). The Ringer's solution was replaced every 2 hours. After completion of epiboly, to avoid the bacterial contamination, the dechorionated embryos were transferred into 0.01% penicillin and 0.01% streptomycin in Ringer's solution and incubated.

Stage definition

Developmental stages were defined morphologically by counting numbers of cells, blastomeres, and somites under a stereomicroscope (Leica M165FC, Germany) and photographed (Leica DFC425C, Germany).

Temperature trial

To describe normal pikeperch embryo development, a control group of 250 embryos with intact chorion were incubated at 15 °C for documentation of timing of embryonic stages as well as fertilization, survival, and hatching rates. Immediately after fertilization, embryos were treated for 2 min with 1.5 ml L^{-1} alcalase to eliminate stickiness, divided into five groups of 50 embryos, rinsed four times in carbon-filtered and aerated tap water, and incubated.

Two experimental groups were created to assess temperature effects. Group A: ~750 eggs were divided into three sub-groups and fertilized, enzyme-treated (as above), and incubated at 10 (Group A1), 20 (Group A2), or 25 °C (Group A3) in carbon-filtered and aerated tap water. Group B: After insemination and enzyme treatment at 15 °C, ~750 eggs were divided into three sub-groups designated B1, B2, and B3 and incubated at 10, 20, or 25 °C, respectively, in carbon-filtered and aerated tap water. Each sub-group was distributed among five 95 mm Petri dishes (n = 50 per dish).

Fertilization rate was calculated at the cleavage stage. Survival rate was determined by the number of fertilized eggs reaching the hatching stage. Hatching rate was calculated over a 72-hour hatching period, determined as the number of surviving embryos.

Statistical analysis

Differences among control and temperature-treated groups in fertilization rate, survival rate, and hatching rate was analysed by the ANOVA and Tukey's HSD test. Differences were considered significant at P-value <0.05. Data are presented as mean ± standard deviation (SD). Analyses were performed using Statistica 13.

Results and discussion

Embryo development in pikeperch at 15 °C

Characterization of the development of the pikeperch embryo was based on morphological features as compared to developmental stages of zebrafish (Kimmel et al., 1995), loach *Misgurnus anguillicaudatus* (Fujimoto et al., 2006), summer flounder *Paralichthys dentatus* (Martinez and Bolker, 2003), ice goby *Leucopsarion petersii* (Arakawa et al., 1999), medaka *Oryzias latipes* (Iwamatsu, 1994, 2006), goldfish *Carassius auratus* (Tsai et al., 2013), and percid species including the rainbow darter *Etheostoma caeruleum* (Paine et al., 1984b), yellow pope *Gymnocephalus schraetser* (Kovac, 1992; Kováč, 1994), yellow perch *Perca flavescens* (Mansueti, 1964), logperch *Percina coprodes* (Cooper, 1978; Paine and Balon, 1984b), walleye *Sander vitreum* (McElman and Balon, 1979), Danube streber *Zingel streber* (Kovác, 2000). Stages were categorized as zygote, cleavage, blastula and gastrula, segmentation, hatching, the free embryo period, initial oral feeding, and larva.

A major feature of sticky eggs, as in pikeperch, is a central lipid drop within the yolk. After egg activation by contact with freshwater, the chorion lifted away and formed a perivitelline space. Pikeperch has the smallest fertilized egg diameter (0.9–1.0 mm) (Güralp et al., 2016) among studied percid species (Kestemont et al., 2015). Fertilization rate was 75% (n = ~5000). Survival rate of fertilized eggs to hatching was 97.2%. Hatching rate of survivors was 92.9% at 15 °C.

Neither cell cleavage nor duration of gastrulation showed similarity with other percid species. Mean time from fertilization to first feeding was 10.2 days. Hatching time was 5.2 days post-fertilization (dpf) at 15 °C, with 5 days post-hatching (dph) to mouth opening.

Blastodisc formation was observed at 1 hpf, and the one-cell stage was observed at 1.5 hpf. The duration of cleavage was 2.5–9 hpf, of blastula 9–21 hpf, of gastrula 21–42 hpf, and of segmentation 42–125 hpf. Hatching began at 125 hpf, and duration was three days. Time of free embryo to first oral feeding was five days. Each period was subdivided into several stages, from fertilization to the end of the hatching period (Table 1).

Table 1. *Developmental stages of pikeperch embryos at 15 °C.*

Stage	Time	Characteristics
Fertilization	0	Perivitelline space appears
Blastodisc formation	1′	Cytoplasm moves to animal pole
Cleavage		
1-cell	1′ 30″	Cytoplasm moves toward animal pole to form the blastodisc
2-cell	2′ 30″	Partial cleavage; 2 equal cells
4-cell	3′ 30″	2 cells in 2 array of cells
8-cell	4′ 30″	4 cells in 2 array of cells
16-cell	5′ 30″	16 cells in 2 array of cells
32-cell	6′ 30″	32 cells in 2 array of double layer
64-cell	7′ 30″	64 cells in 2 array of double layer
Blastula		
128-cell	9′	128 cells in double layer and beginning of YSL forming
256-cell	10′ 15″	256 cells in double layer and single row of YSL
512-cell	11′	512 cells in double layer and irregular YSL
1k-cell	12′	1000 cells in ellipsoidal shape of embryo and E-YSL form
Oblong	13′ 45″	Blastodisc flattening
Sphere	16′ 45″	Spherical shape; border between blastodisc and yolk
Dome	18′ 45″	Shape remains spherical; beginning of epiboly as blastodisc thins
Gastrula		
30% epiboly	21′	Blastoderm an inverted cup of uniform thickness; margin reaches 30% of distance between the animal and vegetal poles
40% epiboly	28′	Germ ring visible at animal pole; margin reaches 40% of distance between the animal and vegetal poles
50%-epiboly	31′	Embryonic shield formation
80% epiboly	32′ 45″	Margin reaches 80% of distance between the animal and vegetal poles
100% epiboly	39′	Complete coverage of the yolk cell by the blastoderm, tail bud
Segmentation		
2-somite	45′	Cephalization begins and 1–2 somite furrow
4-somite	46′	Optic primordium
8-somite	48′	Kupffer's vesicle
12-somite	54′	Furrow shape starts change to chevron
20-somite	62′	Chevron shape and beginning of border between head and body
22-somite	64′	Border between head and body visible, optic lens appears
24-somite	70′	Otolith apparent
28-somite	76′	Tail extension
30-somite	78′	Muscles and median fin fold appear
34-somite	86′	Heart beats, premedium median fin fold
38-somite		Anus formation
40-somite		Straighter longitudinal axis
44-somite	105′	Skin pigmentation
50-somite	117′	Beginning of blood circulation, pigmentation of lens, mouth origination
Hatching		
50-somite	125′	Hatching begins
1 day fry	149′	Yolk sac region is become vessels and skin is more pigmented
1 day fry	154′	Beginning of the pectoral fin formation
1 day fry	160′	Fin fold broader
2 day fry	173′	Red blood cells
2 day fry	183′	Cranium separates the yolk sac; pectoral fin has ~45º angle with lateral body axis, not yet movable
3 day fry	197′	Hatching complete
5 day fry	245′	Mouth opening

Cleavage

Discoidal meroblastic cleavage was characterized by a clear cleavage furrow on the animal pole to generate two equal blastomeres at 2.5 hpf (Fig. 1A). Approximately every subsequent hour, the cells showed divisions from the 2- to 64-cell stage until blastula. The pattern of blastomeres developed from monolayer (1-8 cell stages) to multilayer (16–64 cell stages) rows (Table 1). Cell cleavage duration was 6.5 hours.

Blastula

The blastula period may be separated into early (synchronous) and late (asynchronous) stages, before and after mid-blastula transition, respectively. The synchronous blastula period was signaled by the 128-cell stage at 9 hpf. Many central blastomeres were connected to the yolk cell until the 1k-cell stage at 12 hpf. There was no blastocoel present. The blastodisc had an ellipsoidal shape in the animal polar view at the 1k-cell stage, and the yolk syncytial layer (YSL) could be clearly distinguished. The asynchronous period of blastula consisted of the oblong, sphere, and dome stage (13–19 hpf). The embryo was nearly spherical at the oblong stage, signalling the late blastula period at 13.75 hpf. At 16.75 hpf, the border between the blastodisc and yolk was straight, and the embryo was spherical (Fig. 1D). The internal YSL surface extended toward the animal pole in the center of the blastoderm in the dome stage at 18.75 hpf (Fig. 1E). The duration of the blastula period was 12 hours.

Gastrula

The gastrula period was marked by the beginning of epiboly at 21 hpf, including the formation of a thickened germ ring at the periphery of the blastoderm and the appearance of the embryonic shield. At the epiboly stage, the blastoderm increasingly covered the yolk until completion of epiboly. The germ ring was formed when the margin of blastoderm covered ~40% of the distance between the animal and vegetal poles, at 28 hpf. The dorsal-ventral axis formation was characterized by embryonic shield formation at 50% epiboly, at 31 hpf (Fig. 1F). Differences in gastrula period of pikeperch from other studied teleosts were found. Formation of the germ ring and the embryonic shield occurred at 40% and 50% epiboly, respectively, in pikeperch. In zebrafish, goldfish, and loach, germ ring and embryonic shield are seen at 50% epiboly (Fujimoto et al., 2006; Kimmel et al., 1995; Tsai et al., 2013). The first half of epiboly progressed more slowly than the second half. The embryonic shield elongated toward the animal pole at 80% epiboly (Fig. 1G). The oil droplet was moved to the opposite side of the body. There was no archenteron in the gastrula. At 39 hpf, after yolk plug closure at 100% epiboly, the tail bud had formed, with the oil droplet in close proximity (Fig. 1H). The change in oil droplet position effected rotation of the embryo. This has also been described in S. viterum and P. caprodes semifasciata, which show oil droplet movement associated with cell migration during gastrulation (McElman and Balon; 1979; Paine and Balon, 1984b). The duration of the gastrula period among percids ranges from 6 hours in E. caeruleum to 23 hours in *Z. streber* (Kovác, 2000; Paine and Balon, 1984a). Gastrula in pikeperch was 24 hours, not previously reported in percids, and shows closest similarity to *Z. streber*. Duration of embryonic development of *Z. streber* is 24 days (Kovác, 2000).

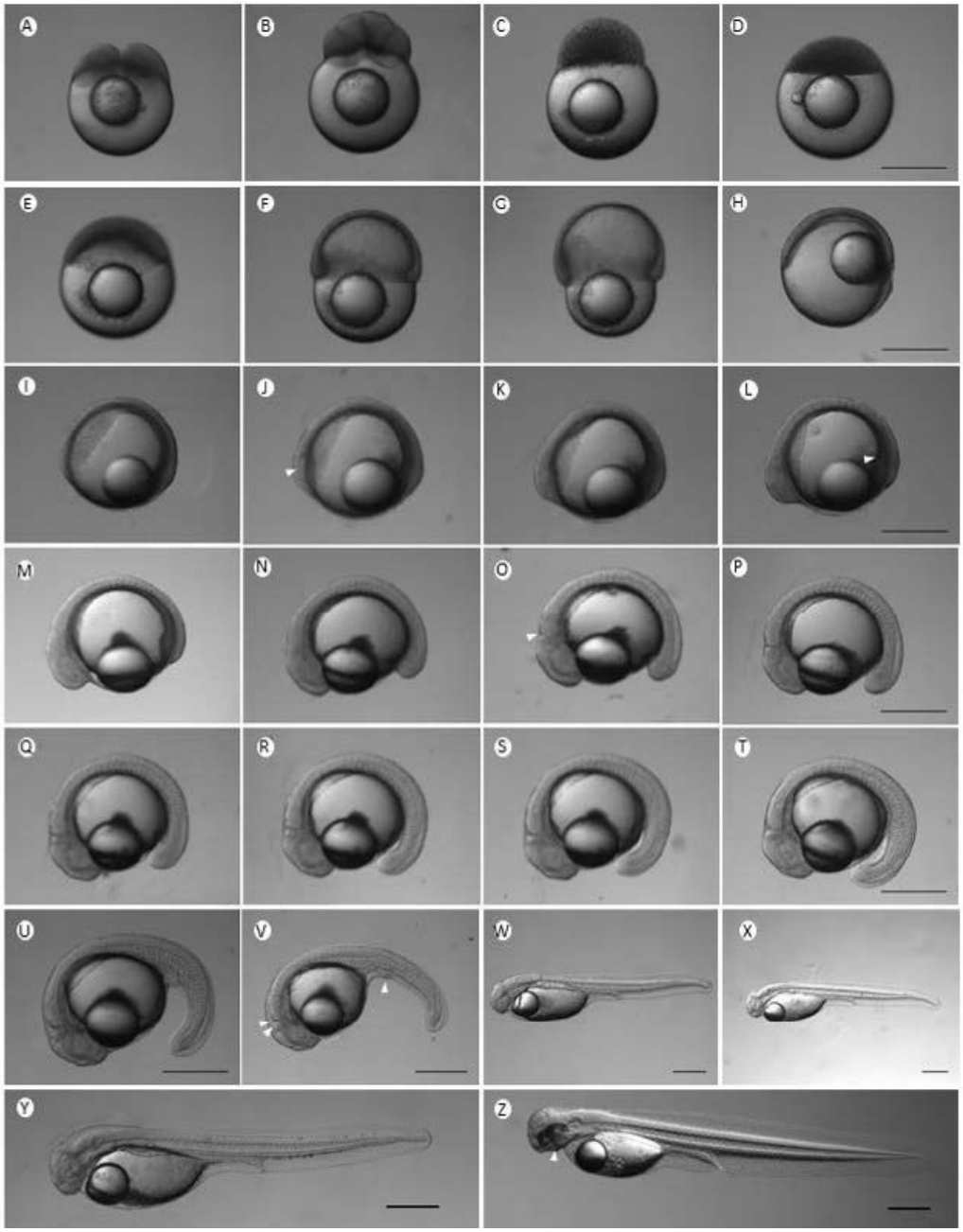


Figure 1. *Embryo development of pikeperch Sander lucioperca at 15 °C.*
A) 2-cell, B) 8-cell, C) 512-cell, D) sphere stage (16 hpf), E) dome-30% epiboly, F) 50% epiboly-germ ring, G) 80% epiboly-embryonic shield, H) 100% epiboly, I) 2- somite, J) 4-somite (optic primordium shown by white arrowhead) K) 6-somite, L) 8-somite (Kupffer's vesicle is shown by white arrowhead), M) 12-somite, N) 18-somite, O) 20-somite, P) 22-somite, Q) 24-somite, R) 28-somite, S) 30-somite. T) 32-somite, U) 34-somite, V) 38-somite W) 40-somite, X) 50-somite Y) 12 hph Z) 24 hph. Scale bar = 500 µm.

Segmentation

This period was recognized at 45 hpf by the appearance of the first somite. The head region became thick, and neurulization was observed with cephalization from the 2-somite stage (Fig. 1I, Fig. 2A, Fig. 3A). The optic primordium appeared at the 4-somite stage (46 hpf) (Fig. 1J, Fig. 2B, Fig. 3B). The eye cups were observed at the 6-somite stage (Fig. 1K, Fig. 2C, Fig. 3C). Kupffer's vesicle formed ventrally in the tail bud region at the 8-somite stage at 48 hpf (Fig. 1L). Somites began to take on a chevron shape after the 12-somite stage, at 54 hpf (Fig. 1M). The tail region bulged and extended through the yolk ball at 18-somites (Fig. 1N, Fig. 4A). At the same time, the oil droplet position shifted from the tail bud region to the (Fig. 1N). This translocation has also been reported in *S. vitreum* (McElman and Balon, 1979). The border between the mesencephalon and hindbrain was clearly visible at the 20-somite stage at 62 hpf (Fig. 1O). These areas were more deeply partitioned at the 22-somite stage (Fig. 1P, Fig. 3G) when optic lens placodes appeared (Fig. 2F, Fig. 3G). Two otoliths were observed in the otic vesicle at the 24-somite stage (70 hpf) (Fig. 1Q, Fig. 2G, Fig. 3H). The tail region began to show separation from the yolk ball at 28-somites (Fig. 1R, Fig. 4E). The body moved with an irregular pulse, the tail region became separated from the yolk and extended toward the posterior, and the median fin fold appeared at the 30-somite (78 hpf) (Fig. 1S, Fig. 4F). Heartbeat began at approximately the 34-somite stage (86 hpf) (Fig. 1U, Fig. 3L) (Movie 1), and a median fin fold became more clear and extended (Fig. 4H). At the 38-somite stage (84.3 hpf), a closed anus was evident Fig. 1V), and the head and anterior body was slightly bent toward the yolk sac (Fig. 3M). The first sustained movements were observed at around the 38-somites stage. The body became almost straight at the 40-somite stage, except for the extreme tail region (Fig. 1W, Fig. 4J). At the 44-somite stage (105 hpf), pigmented cells appeared, and the median fin fold was clearly seen continuous on the dorsal and ventral surface of fish. Embryos had 50 somites at 117 hpf (Fig. 1X).

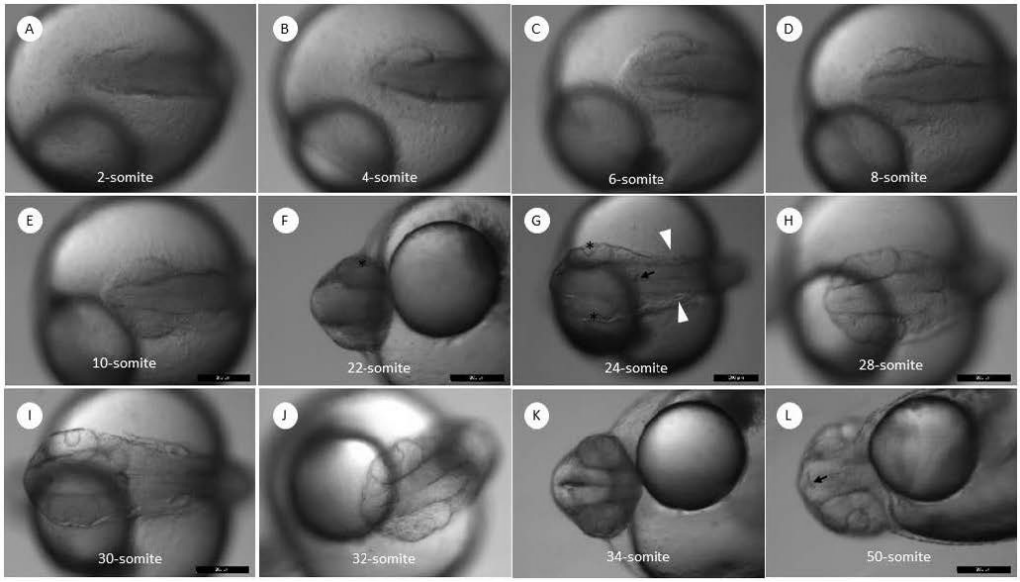


Figure 2. *Dorsal view of head development during pikeperch Sander lucioperca embryogenesis at 15°C.*

A) 2-somite, B) 4-somite, C) 6-somite, D) 8-somite, E) 10-somite, F) 22-somite optic lens is appear (black asterisk), G) 24-somite otolith apparent (white arrowheads), border between mesencephalon and hindbrain (black arrow), optic lenses (black asterisks), H) 28-somite, I) 30-somite, J) 32-somite, K) 34-somite. L) 50-somite mouth origin (black arrow). Scale bar = 200 µm.

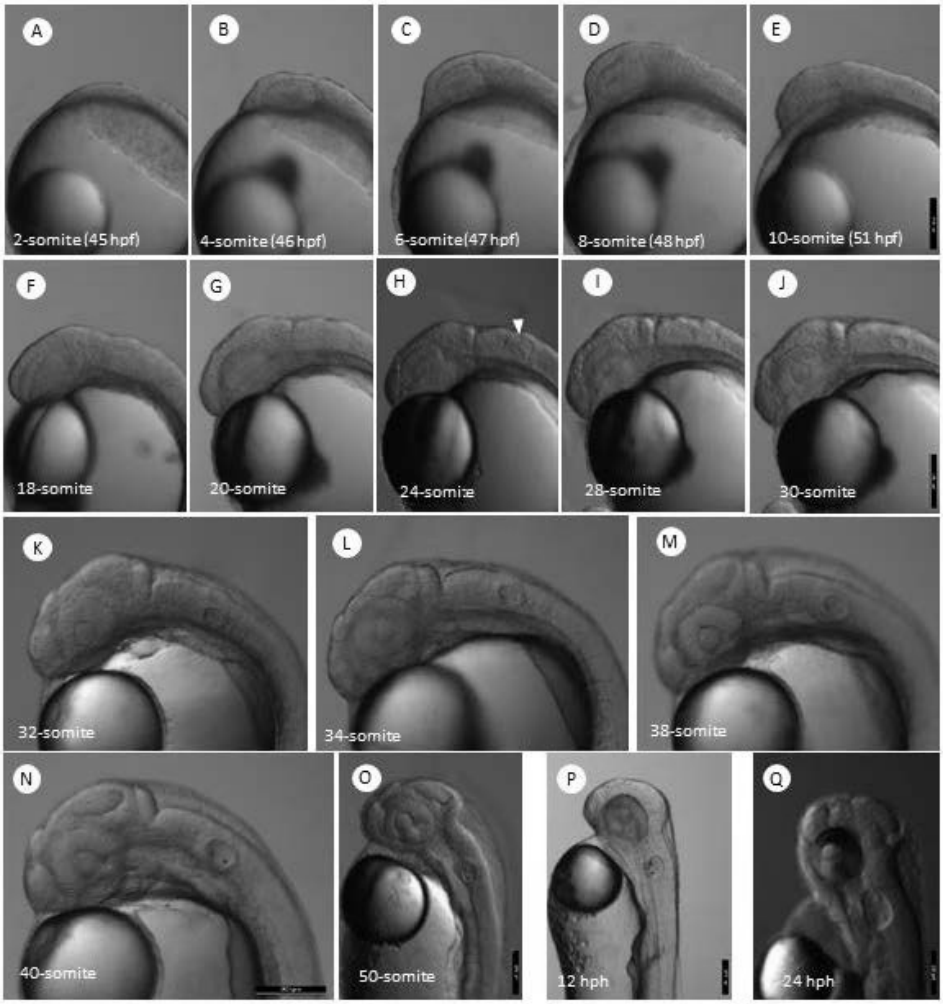


Figure 3. *Lateral view of head development during pikeperch Sander lucioperca embryogenesis at 15 ºC.*

A) 2-somite, B) 4-somite, C) 6-somite, D) 8-somite, E) 10-somite, F) 18-somite, G) 22-somite optic lens apparent, H) 24-somite otolith apparent I) 28-somite, J) 30-somite, K) 32-somite, L) 34-somite. M) 38-somite, N) 40-somite, O) 50-somite P) 12 hpf, Q) 24 hpf. Scale bar = 200 μm.

Video Still 1. *Heart beating. Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.07.050.*

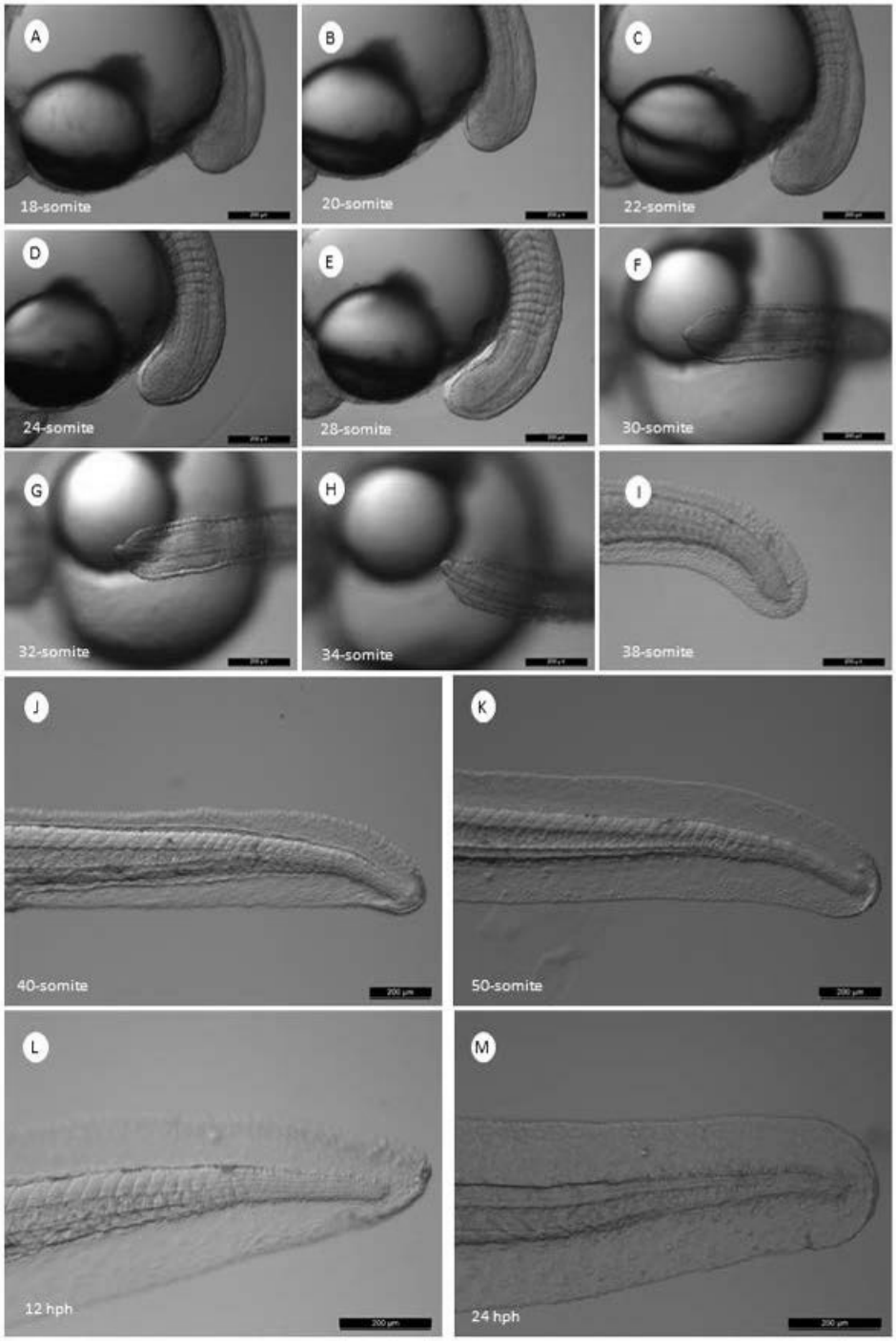
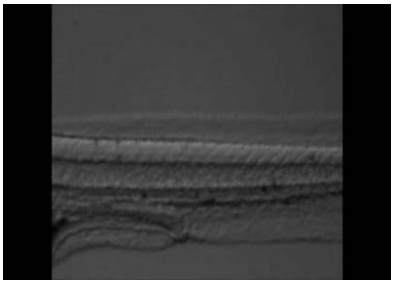


Figure 4. *Tail development during pikeperch Sander lucioperca embryogenesis at 15 °C. A) 18-somite, B) 20-somite, C) 22-somite, D) 24-somite, E) 28-somite tail extension, F) 30-somite, G) 32-somite, H) 34-somite, I) 38-somite, J) 40-somite, K) 50-somite, L) 12 hpf, M) 24 hpf. Scale bar = 200 µm.*

The notochord of straightened, and hatching began at the 50-somite stage (125 hpf), with an asynchronous hatching period of 72 hours. While 12% of embryos hatched at 50-somites, this rate was 44% at 144 hpf, 38% at 168 hpf, and 6% at 192 hpf. Although these results do not confirm reports by Oprea et al. (2014) of a hatching period of 168–240 hpf at 13–16 °C in hatchery conditions, hatching period was three days in both cases. These differences could be due to genetic differences of broodstock or culture conditions, including water temperature.

Although all hatched embryos swam briefly when disturbed, they were usually observed lying still (Fig. 1X). Blood cells were visible running through major vessels (Movie 2). At 149 hpf, the yolk cell was smaller and the body more pigmented, and the mouth appeared (Fig. 1Z, Fig. 2L). The head region was bent toward yolk-sac, and the oil drop within the yolk sac was noticeably smaller. The pectoral fin had ~45° angle with lateral body axis, even though not yet movable at 183 hpf. Pikeperch embryos showed mouth opening at 5 days post hatching (dph). The chronology of embryo pattern and organ development was similar to that in other investigated percids (Schaerlinger and Żarski, 2015), except *P. flavescens* and *E. caerulum*, in which mouth opening was reported to occur before hatching, during segmentation (Mansueti, 1964; Paine and Balon, 1984a).



Video Still 2. *Beginning of blood circulation. Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.07.050.*

The total embryonic period of pikeperch was longer than reported in summer flounder, 85 hours at 14 °C; ice goby, 144 hours at 19 °C; loach, 48 hours at 20 °C; medaka, 82 hours at 27 °C; goldfish, 58 hours at 24 °C; and zebrafish, 48 hours at 28.5 °C (Arakawa et al., 1999; Fujimoto et al., 2006; Iwamatsu et al. 1994, 2004; Kimmel et al., 1995; Tsai et al., 2013). Among percids, pikeperch has one of the shortest embryogenesis periods. There were differences from other teleost fish species in timing of the initial formation of organs, including the optic primordium, Kupffer's vesicle, and otolith (Arakawa et al., 1999; Fujimoto et al., 2006; Iwamatsu et al. 1994, 2004; Kimmel et al., 1995; Tsai et al., 2013). There is no difference among percids in the reported chronology of organ formation and development (Mansueti, 1964; Kováč, 1994; Kovác, 2000; Paine and Balon, 1984a,b; Cooper, 1978; McElman and Balon, 1979). Although the duration of development from fertilization to segmentation of pikeperch is similar to that of *Z. streber* and *P. flavescens*, segmentation duration, timing of hatching, and duration of hatching varies. *Zingel streber* develops in a chorion for 14 days and exists as a free embryo for 10 days (Kovác, 2000), but *P. flavescens* hatches at 26 dpf and exists as a free embryo for three days (Mansueti, 1964). These differences are associated with yolk sac consumption and spawning mode (pelagic or demersal) (Schaerlinger and Żarski, 2015).

A shorter development time may give protection from predators (Mansueti, 1964; Kovác, 2000). The asynchronous hatching period also allows protection from predators or cannibal siblings (Schaerlinger and Żarski, 2015). The hatching period varies from few hours to five days among percids. Individual metabolism, internal resources, and individual differences in oxygen consumption have been shown to be reasons for asynchronous development and hatching in a species (Bang et al., 2004; Kamler, 2007; Régnier et al., 2010). Early pikeperch hatchlings have a shorter body length and more yolk compared to late hatchlings, which tend to develop more slowly and have more yolk remaining around 11–12 dpf (feeding time) (Geffen, 2002; Steenfeldt et al., 2011). Steenfeldt et al. (2011) reported that cannibalism in pikeperch might not be associated with variation in hatching time, but related to high larval growth rate (Kestemont et al., 2007). There is also information that stock density or social hierarchy may have an effect on cannibalism in percids (Baras et al., 2003; Staffan et al., 2002, 2005). To the best of our knowledge, no studies have defined clearly the main reason of the cannibalism in pikeperch.

Effect of temperature on developmental rate

Our results showed adequate development of pikeperch embryos at 10, 15, and 20 °C (Table 2). Among studied percids, *Perca flavescens* has shown tolerance to the broadest temperature range, 10–22 °C. The tolerance to low temperatures may be due to its low spawning temperature of 6.7 °C (Mansueti, 1964). The incubation period of pikeperch is similar to *Gymnocephalus schraetser*, which hatches at 4.2 days post-fertilization (dpf) at 16–20 C (Kováč, 1994) and *Percina caprodes semifasciata*, which hatches at 5.9 dpf at 20 °C (Cooper, 1978; Paine and Balon, 1984b).

No differences in duration of development were observed between the groups fertilized and incubated at a consistent temperature (A1, A2, A3) and those fertilized at 15 °C and subsequently incubated at different temperatures (B1, B2, B3). The first cell formation was observed at 1.25 hpf in the 20 C group, 1.5 hpf at 25 C and 15 °C, and 2 hpf in 10 °C. After first cleavage furrow formation, the cells showed synchronous division until blastula (2- to 64-cell stage) at 1.5 hours at 10 °C, 1 hour at 15 °C, and 45 minutes at 20 °C. The duration of developmental stages increased at lower temperatures (Fig. 5A). The group at 25 °C ceased development within 4 hpf, at the 16-cell stage. Hatching occurred at 176.5, 125, and 69.5 hpf at 10, 15, and 20 °C, respectively (Table 2).

Table 2. *Time (hours' minutes") post-fertilization of developmental stages of pikeperch embryos at four temperature regimes (°C).*

Stage	10	15	20	25
Fertilization	0	0	0	0
Blastodisc formation	1	1	1	1
Cleavage				
1-cell	2	1'30"	1'15"	1'30"
2-cell	3' 30"	2' 30"	2' 0"	2' 0"
4-cell	5' 0"	3' 30"	2 h 45 min	2 h 30 min
8-cell	6' 30"	4' 30"	3' 30"	3'
16-cell	8'	5' 30"	4' 15"	4'
32-cell	9' 30"	6' 30"	5'	
64-cell	11'	7' 30"	5' 45"	
Blastula				
128-cell	13'	9'	6' 30"	
256-cell	14' 45"	10' 15"	7' 15"	
512-cell	16'	11'	8'	
1k-cell	17' 30"	12'	9' 15"	
Oblong	19' 45"	13' 45"	10' 45"	
Sphere	23' 15"	16' 45"	13' 15"	
Dome	27' 30"	18' 45"	14' 15'	
Gastrula				
30% epiboly	33' 15"	21"	15' 15"	
40% epiboly		28'		
50%-epiboly		31'	16' 15"	
70%-epiboly	39' 30"	36'	17' 30"	
80% epiboly			20' 30"	
100% epiboly	46' 30"	39'	21' 30"	
Cephalization	52' 30"	42'		
Segmentation				
2-somite	58' 30"	45'		
4-somite	64' 30"	46'		
8-somite	70' 30"	48'	29' 30"	
12-somite	76' 30"	54'	34'15"	
14-somite	82' 30"			
16-somite	88' 30"			
20-somite	96' 30"	62'		
22-somite				
24-somite	104' 30"	70'		
30-somite	112' 30"	78'	40' 30"	
34-somite	120' 30"	86'	45' 30"	
38-somite	128' 30"		51' 30"	
40-somite	136' 30"			
44-somite	144' 30"	105'	57' 30"	
50-somite	164' 30"	117'	63' 30"	
Hatching	176' 30"	125'	69' 30"	

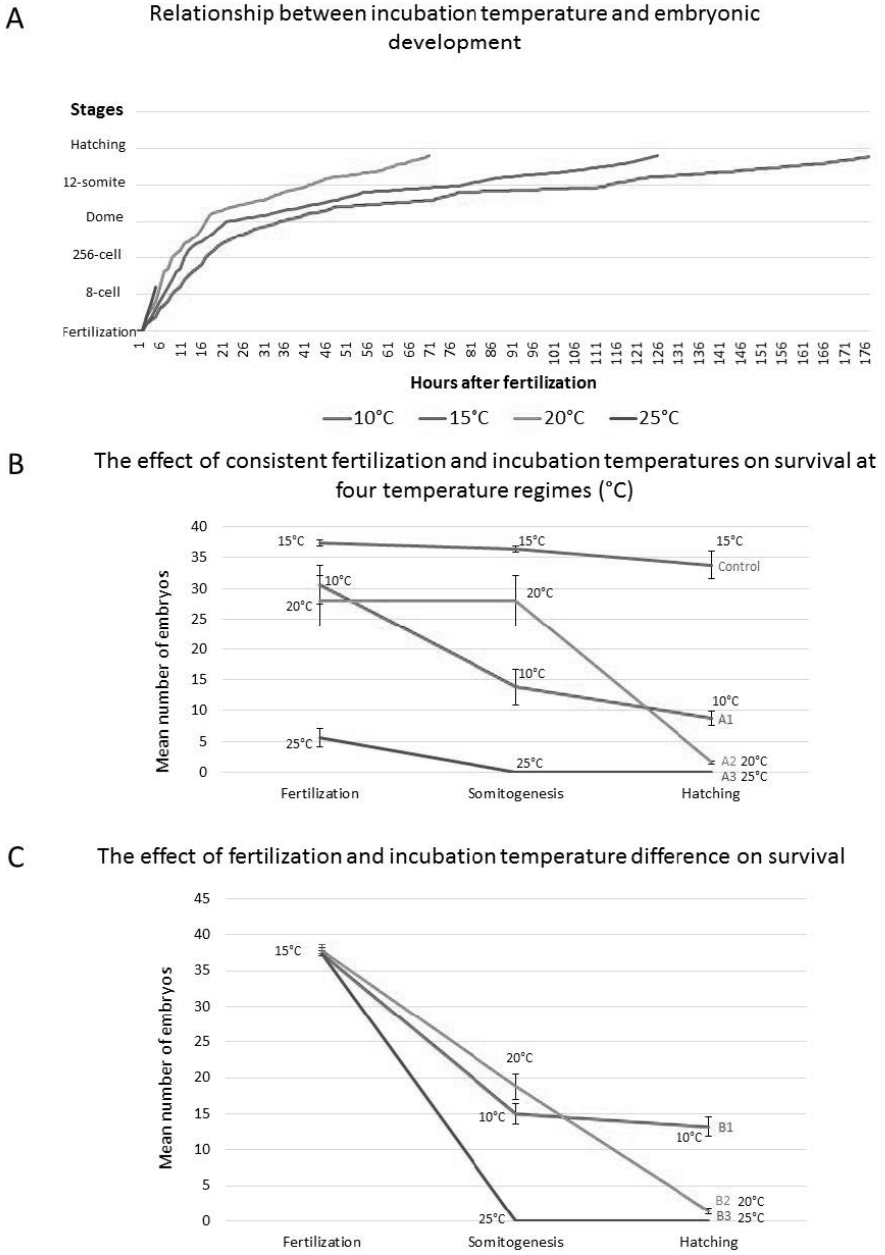


Figure 5. *The effect of the temperature on pikeperch Sander lucioperca development from fertilization to hatching at 10, 15, 20, and 25 °C. A) Relationship between incubation temperature and duration of embryonic development. B) The effect of consistent fertilization and incubation temperatures on survival at four temperature regimes (°C). A1: fertilized at 10 °C, incubated at 10 °C; A2: fertilized at 20 °C, incubated at 20 °C; A3: fertilized at 25 °C, incubated at 25 °C. C) The effect of fertilization and incubation temperature difference of survival. B1: fertilized at 15 °C, incubated at 10 °C; B2: fertilized at 15 °C, incubated at 20 °C; B3: fertilized at 15 °C, incubated at 25 °C. (n = 50, five repetitions for each sub-group of 250 embryos).*

In Group A, fertilization rate at 10 °C (A1) was 14% lower than the controls at 15 °C. There was no significant difference in fertilization rates between group A1 at 10 °C and A2 at 20 °C. Fertilization rate at 25 °C (A3) was significantly lower than groups A1, A2, and controls. Survival rate at 20 °C (A2) was significantly higher than at 10 °C (A1), but hatching rate at 10 °C was significantly higher than at 20 °C. There were no survivors at 25 °C (Table 3, Fig. 5B).

In Group B, with temperature changes to 10, 20, and 25 °C after fertilization at 15 °C, survival rates were significantly lower than control at 15 °C. Survival rate at 20 °C (B2) was 10% higher than at 10 °C (B1), but hatching rate at 10 °C was significantly higher than at 20 °C. There were no survivors at 25 °C (B3) (Table 3, Fig. 5C).

Fertilization rates in Group A at 20 °C (A2) and 25 °C (A3) were significantly lower than the control and Group B at 15 °C (Table 3, Fig. 5B, 5C). In Group B, the survival rate of fertilized eggs was lower than in Group A (Table 3), indicating that survival rate increased when the fertilization and incubation temperatures were the same, although the fertilization rate was consistently low. The highest hatching rate in Group B was observed in B1 at 10 °C (Table 3, Fig. 5C). Incubation at 10 °C was associated with a slower rate of development, which could prove useful for application of surrogate techniques in pikeperch, as it provides a longer manipulation period. If the developmental rate is to be slowed while keeping the survival and hatching rate high, fertilization should be done at 15 °C.

Table 3. *Mean (±SD) survival rate from fertilization to hatching and hatching rate (%) for each treatment at 10, 15, 20, and 25 °C.*

	#	Fertilization (°C)	Fertilization rate (%)	Incubation (°C)	Survival rate of fertilized eggs (%)	Hatching rate of survivors (%)
Control		15	75.0 (0.5)^{a}	15	97.3 (0.5)^{a}	92.8 (2.2)^{a}
Group A	1	10	61.0 (3.0)^{ab}	10	45.0 (2.8)^{c}	63.7 (1.1)^{b}
	2	20	56.0 (4.1)^{b}	20	56.4 (4.1)^{b}	10.1 (0.2)^{c}
	3	25	11.0 (1.5)^{c}	25	0.0^{d}	0.0^{d}
Group B	1	15	75.0 (0.4)^{a}	10	40.1 (1.3)^{c}	88.0 (1.3)^{b}
	2	15	75.0 (0.4)^{a}	20	50.0 (1.7)^{c}	7.4 (0.4)^{c}
	3	15	75.0 (1.1)^{a}	25	0.0^{d}	0.0^{d}

Means in a column with common superscript are not significantly different (ANOVA and Tukey's HSD test, $p < 0.05$) n = 50, five repetitions, 250 embryos per sub-group.

Conclusion

Duration of fish embryo development and their successful hatching depend on environmental factors, including temperature. We described normal development of pikeperch embryos and demonstrated effects of temperature on developmental rate. Pikeperch can be a potential model percid species with flexible incubation temperatures and relatively short embryonic development. The knowledge of similarities and differences among percid species embryogenesis might help to design future studies of surrogate reproduction among percids.

Conflict of interest

The authors declare that no conflict of interest exists among the authors regarding the publication of this paper.

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CHAPTER 3

EARLY EMBRYONIC DEVELOPMENT IN PIKEPERCH (*SANDER LUCIOPERCA*) RELATED TO MICROMANIPULATION

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Early embryonic development in pikeperch (*Sander lucioperca*) related to micromanipulation

H. Güralp, K. Pocherniaieva, M. Blecha, T. Policar, M. Pšenička, T. Saito

South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

ABSTRACT: Recently, transplantation of germ cells has attracted attention as a potential technique for efficient reproduction of fish. One of the well-proven techniques to deliver donor germ cells into a recipient is the transplantation of primordial germ cells (PGCs) during the blastula stage. Nevertheless, the application of such techniques so far has been limited to model fish species such as zebrafish, due to the lack of information about early development in many fish species. We propose that pikeperch (*Sander lucioperca*) can be a useful model species for establishing this technique in the order Perciformes, which includes commercially and ecologically important marine species. In this study, we described the important events, namely, embryonic staging, yolk syncytial layer (YSL) formation, and midblastula transition (MBT) during the blastula stage in pikeperch to obtain basic information about early embryonic development. The chorion was softened by treating with 0.2% trypsin and 0.4% urea in Ringer's solution so as to remove it easily by forceps. Although the first cleavage occurred at about 2.5 h post fertilization, blastomeres divided approximately every one hour after this at 15°C. The YSL was formed after the breakdown of marginal cells during the 512- to 1k-cell stage. Cell division analysis by 4'-6-diaminido-2-phenylindole (DAPI) staining revealed that transition from synchronous to asynchronous division occurred after the 10^{th} cleavage (1k-cell stage). Our results indicate that zygotic gene expression (MBT) starts after this stage. Next, we performed blastodisc isolation assay to find the competent stage for embryonic manipulation. Embryos were manipulated by using a microneedle every hour from the 512-cell to the sphere stage, and then developmental rates were evaluated at the hatching stage. The highest survival rate was obtained when we performed this manipulation at the 1k-cell stage. These results clearly showed that the MBT is the best stage for transplantation of PGCs or any cells in pikeperch.

Keywords: blastodisc isolation; blastula; cleavage; early embryogenesis; yolk syncytial layer; midblastula transition

INTRODUCTION

Recent advances in the technology of germ cell transplantation have introduced new avenues of research in fish reproductive management for aquaculture. Transplantation of germline stem cells (GSC) provides an increased supply of donor-derived gametes for surrogate hosts. The fish-seed production is expected to become more efficient by the realization of surrogate production between two different species that have different biological characteristics (Yamaha et al. 2007). For example, the life cycle of the fish might be shortened if a species that has shorter life cycle is used as the surrogate host and produces donor gametes. Moreover, the resources required for fish culture

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(i.e. space, water, food, fuel) might be reduced if a species that has small body size is used as the host for producing gametes of species with a bigger body size. A key factor for implementation of the surrogate production in fish is how to incorporate donor germ cells into the host gonad. Yamaha et al. (2003) transplanted primordial germ cells (PGCs) of crucian carp (*Carassius carassius*) into embryos of the domesticated goldfish (*C. auratus*) and obtained donor-derived gametes from the matured "germline chimeras" (Yamaha et al. 2003). Saito et al. (2011) have shown that a single PGCs transplantation technique works even between marine and freshwater species. Okutsu et al. (2007) have also produced germline chimeras by transplanting rainbow trout (*Oncorhynchus mykiss*) spermatogonia from adult testis into sterile hatched larvae of masu salmon (*O. masou*), and they clearly showed that the germline chimeras generated only gametes of the donor. On the other hand, a transplantation technique has been developed both in tilapia and atherinopsid species, respectively, where spermatogonia from a donor were inserted directly into an adult recipient testis via genital pore (Majhi et al. 2009; Lacerda et al. 2010). However, these aforementioned techniques have also drawbacks (see the review by Robles et al., submitted). To start with the case of PGCs transplantation as shown by Yamaha et al. (2003) and Saito et al. (2011), the manipulation must be performed in a short period during the embryonic stage. In addition, it is difficult to prepare PGCs in the amount appropriate for transplantation. Regarding the spermatogonia transplantation by Okutsu et al. (2007), Majhi et al. (2009) or Lacerda et al. (2010), although numerous cells can be prepared from an adult fish, the efficiency of germline chimerism is low.

The most widely used technique due to its methodological simplicity is blastomere transplantation (BT) (Lin et al. 1992; Wakamatsu et al. 1993; Takeuchi et al. 2001; Yamaha et al. 2001; Nakagawa et al. 2002; Saito et al. 2011). In many fish species, the PGCs localize around the marginal region of the blastodisc during blastula stage (Yoon et al. 1997), and it is difficult to distinguish the PGCs from the somatic blastomeres. Thus, the blastomeres at the marginal region are randomly aspirated into a micro-glass needle and transplanted into a host embryo at the same stage. The confirmation of germline chimerism after BT can be performed by observation of the donor's genetic markers in chimeras' offspring, or by visualization of PGCs with PGCs-labelling molecules as shown by Saito et al. (2011).

The applicability of the BT technique is based on the nature of blastula stage embryo. Blastomeres are pluripotent in their ability to differentiate into any cell types except PGCs, and they last until gastrula. The fate of PGCs has been specified by inheritance of germplasm. Thus, the transplanted blastomeres are capable of contributing to the development of recipient without mismatches of tissue affinity that can cause deformation of the embryo (Saito et al. 2011). In addition, donor-derived PGCs are able to migrate toward the genital ridge of a host embryo at high efficiency after BT (Saito et al. 2011). However, in a series of blastoderm transplantation experiments, the blastomeres lost multipotency and tolerance to surgical manipulation gradually disappeared as the embryo developed (Yamaha et al. 1998).

The loss of blastomere multipotency coincides with mesoderm and endoderm formation. These embryonic layers are initially induced at the equator of the embryo by signals arising from the yolk syncytial layer (YSL) after a midblastular transition (MBT) stage (Mizuno et al. 1996; Chen and Kimelman 2000). Thus, the YSL formation and MBT stages are critical initial steps in embryonic development. From this point of view, the understanding of developmental changes in the blastula stage is important in order to apply the BT technique in the species to be propagated. However, there are restrictions in only some fish species according to detailed information about both BT technique applicability and embryonic development during blastula stage (Yamaha et al. 1998).

Pikeperch, *Sander lucioperca,* is one of the most important freshwater aquaculture species in many European countries such as the Czech Republic, Denmark, Hungary, Romania, Tunisia, Ukraine, the Netherlands, and Poland (FAO 2015). Pikeperch belongs to the order Perciformes, which includes numerous commercially and ecologically important species. In this study, we described the early embryonic development of pikeperch in details during cleavage up to blastula stages. Then, we performed "blastodisc isolation assay" to determine the best period for micromanipulation of BT transplantation during blastula stage. We

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propose that pikeperch is a suitable candidate as a recipient species for applying surrogate production technology in the Perciformes.

MATERIAL AND METHODS

Ethics. All experimental procedures were performed in accordance with the National and Institutional guidelines on animal care and experimentation. This study was approved by the Animal Research Committee of the University of South Bohemia in České Budějovice.

Fish and preparation of embryos. Fish were kept in the aquaculture facility of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in Vodňany, Czech Republic during spawning season from March to April in 2013 and 2014. During handling, the fish were anesthetized with clove oil (0.03 ml/l water) (Dr. Kulich Pharma, s.r.o., Hradec Králové, Czech Republic). Ovulation and spermiation were induced by the injection of human chorionic gonadotropin (Chorulon) (Kristan et al. 2013). Eggs were obtained from two different females, and these were fertilized with sperm from three males in filtered and aerated tap water at 15°C.

Dechorionation of embryos. Fertilized eggs were treated for 5–10 min with 0.2% trypsin and 0.4% urea in Ringer's solution (128mM NaCl, 2.8mM KCl, 1.8mM $CaCl_2$ $2H_2O$) buffered by 10mM TAPS to pH 8.5 to remove the stickiness and soften the chorion (Saito et al. 2011). The chorion was manually removed using fine forceps and the dechorionated zygotes were maintained until the completion of epiboly in Ringer's solution buffered by 10mM HEPES to pH 7.5, containing 1.6% albumin on 1% agar-coated dishes. Embryos with intact chorions were cultured in filtered and aerated tap water in individual plastic Petri dishes (diameter 120 mm). Ringer's solution was replaced every 2 h. After the completion of epiboly, the dechorionated embryos were transferred to a 2nd culture solution (1.8mM $MgCl_2$ and 1.8mM $CaCl_2$) containing 0.01% penicillin and 0.01% streptomycin and incubated at 15°C.

Stage definitions. During cleavage period, embryos incubated at 15°C were observed every 15 min under a stereomicroscope Leica M165FC (Leica Camera AG, Wetzlar, Germany) and photographed by a Leica camera DFC425C. Developmental stages were examined for morphological characteristics such as numbers of blastomeres and a shape of the embryo.

Visualization of nuclei for MBT staging. To plot a ratio of embryos with cells that showed anaphase or metaphase cycle at each time point, nuclei of embryos were visualized and observed. More than twenty embryos were fixed every 15 min until 15.5 h post-fertilization (hpf) with Farmer's fixative (75% ethanol and 25% acetic acid) and kept at 4°C overnight, then replaced with 100% methanol and stored at –20°C until analysis. After dechorionation, blastodiscs were removed from the yolk, and the nuclei were stained with 5% 4'-6-diaminido-2-phenylindole (DAPI) in phosphate-buffered saline (PBS) for 10 min. Then, embryos were washed in PBS and cell divisions in embryos were observed under an inverted fluorescence microscope Olympus IX83 (Olympus, Tokyo, Japan) and photographed with a digital camera C10600 ORCA-R2 (Hamamatsu Photonics, Hamamatsu, Japan).

Blastodisc isolation assay. A blastodisc isolation assay was performed so as to find an optimal stage for embryonic manipulation as described by Yamaha et al. (1998); dechorionated embryos were examined at one-hour intervals from 12 hpf to 17 hpf (512-cell to sphere) (Figure 4A). Then, the developmental rate of each experimental group was evaluated at hatching (Figure 4B). Manipulation was performed in Ringer's solution buffered by 10mM HEPES to pH 7.5 containing 1.6% albumin on 1% agar coated dish. Blastodisc was separated from the yolk mass using a fine glass needle, then replaced in the same position. The manipulated zygotes were incubated in the same dish for 30 min to allow healing of the disrupted cells; zygotes were then placed separately in a 96-well culture dish. At least 18 manipulated zygotes were produced for each stage. During mid-somitogenesis stage, manipulated embryos were transferred into the 2nd culture solution and cultured until hatching. We evaluated survival and malformation rates by observing them under the stereomicroscope.

RESULTS

Early embryonic development in pikeperch. The embryos had a single oil globule within the yolk. The mean diameter of the oil globule was 482.4 µm, and it was about one-half of the yolk diameters (mean: 919.4 µm). The oil globule was located in the upper-central area of the yolk until gastrulation (Figure 1). The animal pole and the

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blastodisc faced downward toward the bottom of the dish after gastrulation but the oil globule, which was not fixed in the yolk mass, moved upward toward the vegetal pole.

The pikeperch embryos have meroblastic discoidal cleavage (Figure 1). The first division occurred around 150 min post fertilization (mpf) at 15°C. Then at approximately one-hour intervals the zygotes cleaved synchronously until the blastula period. The blastomeres formed in a monolayer until the 8-cell stage, and then formed a multilayer from 16-cell stage (Figure 1E–L). Periodic cleavages were observed until around the 512-cell to 1k-cell stage by serial photographs: all blastomeres divided into two daughter cells simultaneously. It was not possible to estimate the number of blastomeres beyond the 1k-cell stage because each blastomere became smaller with each division and the changes in the embryo shape were either slight or indistinguishable until the sphere stage

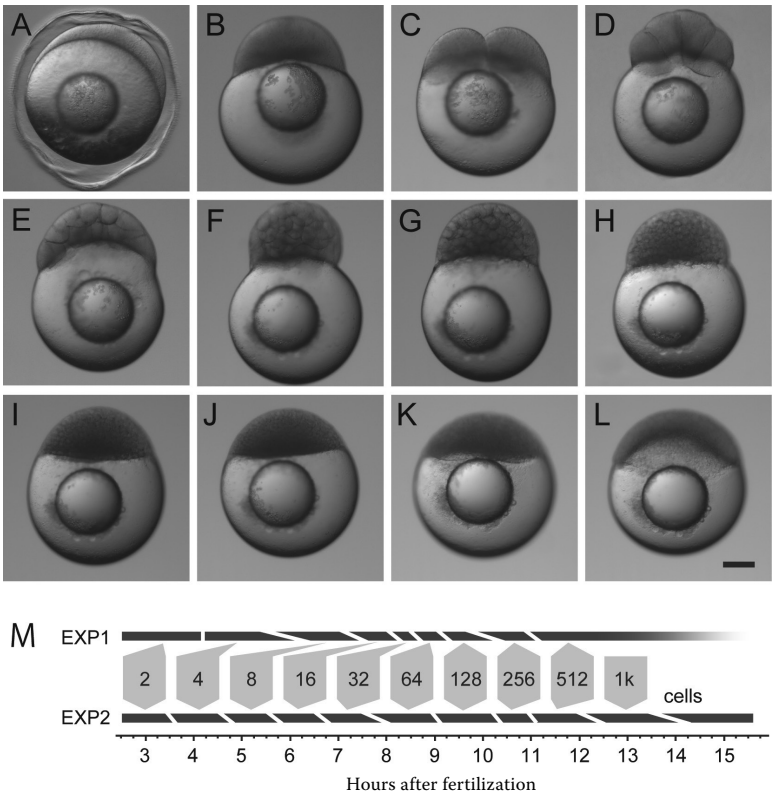

Figure 1. The first 18 hours of pikeperch embryo development

(**A**) 5 min after fertilization; (**B**) 1-cell stage; (**C**) 2-cell stage; (**D**) 8-cell stage; (**E**) 16-cell stage; (**F**) 32-cell stage; (**G**) 128-cell stage; (**H**) 512-cell stage; (**I**) 1k-cell stage; (**J**) 15 hours post fertilization (hpf) blastula; (**K**) 17 hpf blastula (sphere stage); (**L**) dome stage. Note that after the 512-cell stage till the sphere stage (11–17 hpf), the morphological change of embryos is very small. Although embryos become spherical in shape gradually during this period, the change is continuous, and the stage cannot be divided according to the shape; (**M**) developmental speeds varied by batch and embryos. Embryos observed in Experiments 1 and 2 were obtained from different parents in a different season, respectively. 10 embryos were observed for staging in each experiment. White lines on blue bars show the transition of each stage. At the time period of white lines, some embryos showed an advanced stage while some showed a previous stage simultaneously. In the case of Experiment 1, we could not distinguish stages following the 512-cell due to a very slight change in embryo shape. The scale bar indicates 200 μm

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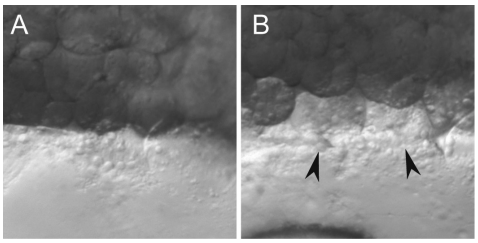

Figure 2. Yolk syncytial layer formation in a pikeperch embryo

(**A**) majority of blastomeres located at the marginal region showed round shape at the 512-cell stage; (**B**) many marginal cells collapsed onto the yolk cell at the 1k-cell stage (arrowheads). The collapsed blastomeres merged into a layer after this stage

(Figure 1I and 1J). The border between blastodisc and yolk was not elevated, and the embryo exhibited a spherical shape at 16.7 hpf (Figure 1K). The embryos became dome-shaped at 18.7 hpf (Figure 1L). It is important to note that there was variation in developmental rate among batches from different parents (Figure 1M). All experiments henceforth were performed using Experiment 2 embryos. During 512-cell to 1k-cell stages, the marginal cells collapsed onto the yolk surface and after this stage, the yolk syncytial layer (YSL) was formed at the marginal zone of the blastodisc (Figure 2).

Mid-blastula transition (MBT). Although there were slight differences in the developing speed among embryos, the majority of the embryos developed synchronously, and the plot graph revealed the general time course of the cell division pattern of the pikeperch embryos (Figure 3A). The time schedule of this synchronous cell cycle corresponded with the observations under the microscope (Figure 3A). The blastomeres divided synchronously up to 1k-cell stage (13.25 hpf) (Figure 3B), and then after this stage (13.5 hpf) the divisions were asynchronous, and cells with the anaphase or metaphase cycle were observed in almost all embryos (Figure 3B). These results clearly showed that the mid-blastula transition started after 1k-cell stage.

Blastodisc isolation assay. In the control group, 95.0% embryos (n = 40) developed and hatched normally. The highest survival rate (73.6%) in the experimental groups was at the 1k-cell stage (n = 19) (Figure 4C), suggesting that this stage is the best for manipulation. Although the survival rates were above 50% in the group of 512-cell (n = 19) and 14 hpf (n = 18), the rates of abnormal development were slightly higher at these phases than

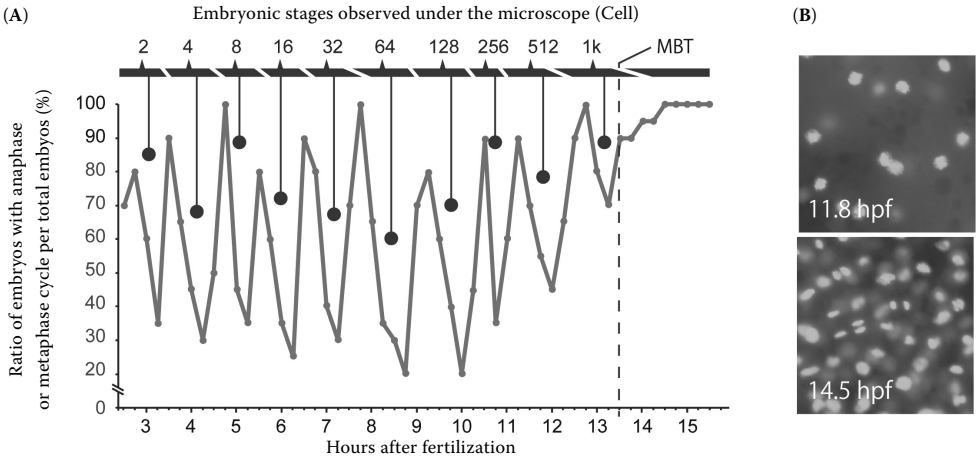

Figure 3. Transition from synchronous cleavage to asynchronous in a pikeperch embryo

(**A**) plots of embryos that have cells in anaphase or metaphase per total embryos showing periodical cycles of cleavage until the 1k-cell stage. After 13.5 h post fertilization (hpf), embryos lost synchronous cell cycles and the mid-blastula transition (MBT) started; (**B**) typical image of synchronous cleavage (above, 11.8 hpf) and asynchronous cell division (below, 14.5 hpf). In the 14.5 hpf embryo, various phases of cell division are observable

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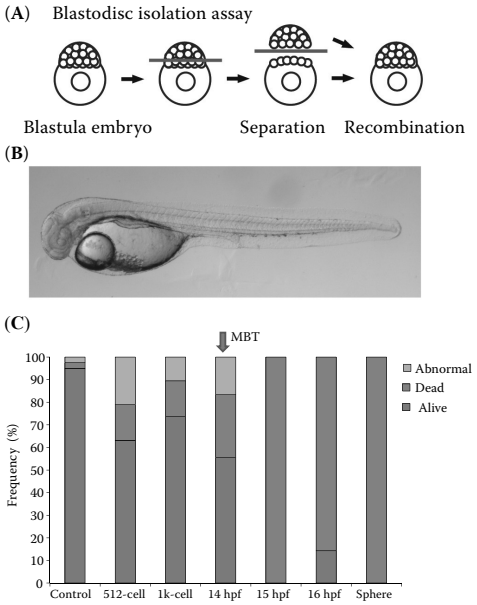

Figure 4. Blastodisc isolation (BI) assay

(**A**) schematic illustration of the BI-assay. Blastodisc was isolated by a micro glass needle and then recombined onto the yolk again. By this assay, we can assess the general reaction against manipulation in each stage; (**B**) hatching stage embryo; (**C**) developmental rate after BI-assay in each stage from 512-cell (11 h post fertilization (hpf)) until 15 hpf was above half the population of the experimental groups. After mid-blastula transition (MBT), some survivals can be detected only at 16 hpf

that of the 1k-cell stage. Almost all manipulated embryos in the 15–17 hpf groups (15 hpf n = 14, 16 hpf n = 14, 17 hpf (sphere) n = 17) showed abnormal shape during development, and they did not survive to hatching.

DISCUSSION

In this study, we described the early embryonic events in pikeperch embryos that encompass the most competent stage for transplantation; this can be considered as basic information for beginning PGCs transplantation in the Perciformes.

The staging of embryonic development in pikeperch has only been described briefly in general terms such as external morphology (Schlumberger and Proteau 1996; Oprea et al. 2014). However, description of the blastula stages during cleavage has not been presented until now in pikeperch. Unfortunately, the staging information obtained for other species based only on the external characteristics cannot be directly applied to pikeperch. In the past two decades, developmental processes during blastulation in fish have been described mainly in cyprinid species, such as zebrafish (Kimmel et al. 1995), loach (Fujimoto et al. 2004), and goldfish (Yamaha et al. 1999; Tsai et al. 2013). Important developmental events during early development can be summarized according to these papers into the following three categories: (1) increasing number of blastomeres by synchronized cell division; during this period, cell number can be counted, (2) yolk syncytial layer (YSL) formation as a preliminary step for germ layer and dorsal axis patterning, and (3) transition from the synchronous to asynchronous cell division, which is known as the mid-blastula transition (MBT). The pattern of germ layer succession can be described using these three transitional phases. We classified these three events as the result of this study as follows:

(1) Synchronized cell division period: 1- to 1k-cell stage. Synchronized cell cycles were repeated 10 times until 13.5 hpf at the culture condition of 15°C. This cycle is identical with that of zebrafish (Kane and Kimmel 1993). However, in goldfish, it is repeated 9 times (Yamaha et al. 1998).

(2) yolk syncytial layer (YSL) formation: In pikeperch, the marginal blastomeres broke down during the 512- to 1k-cell stages and thereafter, nuclei from these blastomeres formed YSL. This sequence is identical to that of zebrafish where the marginal cells undergo a collapse and release their cytoplasm and nuclei by the 512-cell stage (Kimmel and Law 1985). It has been reported in zebrafish and goldfish that the yolk mass with the YSL provides signals that induce the mesoendoderm and the dorsal axis to the pluripotent blastodisc after the mid-blastula stage (Mizuno et al. 1996; Yamaha et al. 1998; Chen and Kimelman 2000). Furthermore, YSL is necessary for epiboly (Kimmel et al. 1995). In this manner, YSL plays a central role in the germ layer patterning and morphogenesis in fishes and is important to understand when this structure is formed during development. The formation of YSL in a pikeperch embryo will be a good marker for staging.

(3) Mid-blastula transition (MBT): A period of transition from synchronous to asynchronous

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cell division is known as MBT. In *Xenopus* and zebrafish, it has been known that zygotic gene expression and active cell migration begin at this stage (Newport and Kirschner 1982; Kane and Kimmel 1993). In pikeperch, asynchronous cell division began after 13.5 hpf (after 1k-cell stage) at 15°C, so this stage is probably the MBT in pikeperch.

In pikeperch embryo, the two events (YSL formation and MBT) occuring around 12–14 hpf related to blastodisc isolation assay demonstrated that embryos have the high competency for manipulation just before ending of blastula period. As a matter of fact, previous studies have shown that the blastomeres remain pluripotent and uncommitted throughout the late blastula and early gastrula stages (Ho and Kimmel 1993; Yamaha et al. 1998). Furthermore, in the present study, embryos had different levels of sensitivity ranges to blastodisc separation comparatively in 512-cell, 1k-cell, and 14 hpf stages. Interestingly, the hatching rate of the 512-cell stage group was slightly lower than that of the 1k-cell stage. At the 512-cell stage, the blastomeres were comparatively larger than those of later stage. It seems that big blastomeres are fragile to manipulation. In fact, blastodisc separation was quite difficult at the 256-cell stage because many blastomeres were destroyed with the glass needle. Thus, it is reasonable to suggest that broken cells affected the development of embryos at the 512-cell stage. On the other hand, embryos after 15 hpf were very sensitive to blastodisc separation, and the developmental rate was quite low. Generally, after MBT the embryo gradually becomes more sphere-shaped, and the tension of the surface of embryos increases as it develops, together with the beginning of the germ layer patterning. This embryonic movement might hinder recovering of a fresh wound after manipulation. Nevertheless, we showed that the optimal stage for micromanipulation in pikeperch embryos is 12–14 hpf at 15°C. This information can be used for PGCs transplantation for pikeperch in the future.

In this study, we described the critical events in early embryonic development and competent stages for micromanipulation in pikeperch embryos. This study suggests that germline chimera can be produced in pikeperch by PGCs transplantation during the blastula stage. The family Percidae includes some important aquaculture species, some are freshwater while others live in seawater. Our results may be applicable to surrogate production techniques in pikeperch such as generation of chimeras between freshwater and seawater species by transplantation of germ cells (Saito et al. 2011). By using pikeperch as a host, many biotechnological applications might be possible in the reproduction of Perciformes fish species.

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Corresponding Author

Hilal Güralp, MSc., University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic
Phone: +420 776 719 572, e-mail: guralh00@frov.jcu.cz

CHAPTER 4

MIGRATION OF PRIMORDIAL GERM CELLS DURING LATE EMBRYOGENESIS OF PIKEPERCH *SANDER LUCIOPERCA* RELATIVE TO BLASTOMERE TRANSPLANTATION

Güralp, H., Pocherniaieva, K., Blecha, M., Policar, T., Pšenička, M., Saito, T., 2017. Migration of primordial germ cells during late embryogenesis of pikeperch *Sander lucioperca* relative to blastomere transplantation. Czech J. Anim. Sci. 62, 121–129.

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Migration of Primordial Germ Cells During Late Embryogenesis of Pikeperch *Sander lucioperca* Relative to Blastomere Transplantation

Hilal Güralp, Kseniia Pocherniaieva, Miroslav Blecha, Tomáš Policar, Martin Pšenička, Taiju Saito*

Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

**Corresponding author: guralh00@frov.jcu.cz*

ABSTRACT

Güralp H., Pocherniaieva K., Blecha M., Policar T., Pšenička M., Saito T. (2017): **Migration of primordial germ cells during late embryogenesis of pikeperch *Sander lucioperca* relative to blastomere transplantation**. Czech J. Anim. Sci, 62, 121–129.

Pikeperch *Sander lucioperca* is a valuable fish in Europe, and basic information about its embryonic development, especially primordial germ cell (PGC) migration, is important for use in biotechnology. We categorized pikeperch embryonic development into six stages as in other fish species: zygote, cleavage, blastula, gastrula, segmentation, and hatching and described PGC migration. PGCs were visualized by injection of synthesized green fluorescent protein (GFP) within the 3'untranslated region (UTR) mRNA of *nanos3*. GFP-positive PGCs appeared in all embryos at approximately 100% epiboly. Time-lapse imaging revealed the PGC migration pattern from their initial appearance to location at the gonadal ridge. We conducted blastomere transplantation (BT) at the blastula stage. Donor embryos were labelled with GFP-*nos3* 3'UTR mRNA and tetramethylrhodamine dextran to label PGCs and somatic cells, respectively. Twelve BT chimeras were produced, with eight surviving to hatching. All exhibited donor-derived somatic cells in the developing body. The PGCs from donor embryos were observed to migrate towards the gonad region of the host embryos. Our results indicated that BT can be successfully applied in pikeperch, and these findings may be useful to produce germline chimeras in percids.

Keywords: germ cell transplantation; germ cell migration; germ-line chimera; *nanos*; primordial germ cell visualization

Pikeperch *Sander lucioperca* is a valuable recreational and commercially important freshwater fish for inland European aquaculture (Hilge and Steffens 1996). It belongs to the order Perciformes, which also includes numerous commercially and ecologically important marine species (Nelson 2006). The scientific community has recently shown interest in surrogate reproduction of fish using

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germ cell transplantation technology (GCTT) (Takeuchi et al. 2001; Yamaha et al. 2003; Okutsu et al. 2007; Saito et al. 2008, 2011). The gametes of a donor fish can be obtained via a germ-line chimera after germline stem cell transplantation. Germ cell transplantation provides benefits, including reducing the time for fish to reach sexual maturity and the space required for aquaculture (Psenicka et al. 2015), banking the genetic resource by cryopreservation of germ cells (Linhartova et al. 2014; Psenicka et al. 2016), producing fish by *in vitro* cultivation of germ cells without maintaining broodfish (Shikina et al. 2013), and the possibility of producing marine fish on land. Keeping marine fish in captivity generally requires costly equipment, such as large cages and nets, not used for freshwater species. Culture of marine fish on land is expensive, since maintaining relatively small volumes of seawater in a stable condition, with the salt concentration affected by evaporation, is a challenge. It may be of value to reproduce marine fish species in freshwater using surrogate breeding. However, the only report on successful production of a germ-line chimera in fish of different habitats is GCT between the catadromous Japanese eel *Anguilla japonica* and the freshwater zebrafish *Danio rerio* (Saito et al. 2011).

A germline chimera can be generated through spermatogonia transplantation into larvae (Lacerda et al. 2013). However, pikeperch larvae are sensitive and cannot survive transplantation or even mild anesthesia (Guralp, unpublished data). Transplantation of blastomeres (BT) containing PGCs is probably the only method for the generation of a germ-line chimera that can be successfully applied in pikeperch as our previous work has demonstrated (Guralp et al. 2016). However, the contribution of donor PGCs to the host embryo is an issue that needs to be addressed, as the donor-derived cells must be incorporated into the developing embryo. Knowledge of embryonic development is crucial to the assessment of PGC contribution to the recipient embryo. Lack of basic information on germ cell development during embryogenesis in pikeperch impedes the application of such surrogate production techniques. The present study focused on characteristics of late embryogenesis.

The origin and migratory pathways of PGCs vary among animals (Richardson and Lehmann 2010; Saito et al. 2014) and are not identical even in teleosts (Saito et al. 2006). The origin and migration pathway of PGCs have been studied in fish species including zebrafish, loach *Misgurnus anguillicaudatus*, goldfish *Carassius auratus*, medaka *Oryzias latepis*, Pacific herring *Clupea pallasii*, ice goby *Leucopsarion petersii*, tench *Tinca tinca* (Weidinger et al. 1999; Fujimoto et al. 2006; Saito et al. 2006; Linhartova et al. 2014), sturgeon *Acipenser* spp. (Saito et al. 2014), and flounder *Paralichthys olivaceus* (Li et al. 2015; Wang et al. 2015). The PGC migration pattern can vary, even in species within a family, as Saito et al. (2008, 2010) reported for Cyprinidae. Nevertheless, it has recently been revealed that germline chimeras can be produced between distantly related family members occupying different habitats (catadromous and freshwater). Japanese eel PGCs migrate and localize to the genital ridge of the zebrafish embryo after transplantation, although donor-derived PGCs disappear during the development of the chimeras (Saito et al. 2011). The pattern of PGC migration during late embryonic development of percids is unclear.

The PGCs can be visualized beginning at the mid-gastrula stage of embryogenesis by injecting synthetic mRNA constructed with a green fluorescent protein (GFP) sequence within the 3' untranslated region (UTR) of RNA of *vasa* or *nanos3* into 1–2 cell stage embryos (Koprunner et al. 2001; Yoshizaki et al. 2005; Saito et al. 2006; Linhartova et al. 2014). Saito et al. (2006) labelled PGCs in six species by injecting GFP-*nos3* 3'UTR zebrafish mRNA and found its function to be conserved.

The goal of this study was to use GFP labelling to describe the migration of PGCs from gastrula through hatching, and to evaluate the potential of BT in pikeperch. The results will provide needed information on generating germline chimeras in pikeperch.

MATERIAL AND METHODS

Ethics. The maintenance of fish and experimental procedures were conducted according to the criteria of the EU Harmonized Animal Welfare Act of the Czech Republic, Act No. 246/1992 Coll.

Fish and preparation of embryos. Pikeperch were obtained from the recirculating aquaculture system at the University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of

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Waters during the spawning season, March through April, 2013 and 2015. The fish were anesthetized with clove oil at 0.03 ml/l water (Dr Kulich Pharma, s.r.o., Czech Republic). Ovulation and spermiation were induced by the intramuscular injection of human chorionic gonadotropin (Chorulon; Merck, USA) (Kristan et al. 2013). Eggs and sperm were collected from three females and three males ~ 83 d° after the hormone treatment. Eggs were inseminated with the sperm in filtered and aerated tap water at 15°C. Fertilized eggs were treated for 10 min with 0.2% trypsin and 0.4% urea in Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$) buffered by 10 mM TAPS to pH 8.5 to remove stickiness and soften the chorion. After enzyme treatment, embryos were individually dechorionated using fine forceps. The dechorionated embryos were divided into ten 1% agar-coated Petri dishes (diameter 95 mm) with each dish containing up to 50 embryos and incubated in Ringer's solution buffered by 10 mM HEPES to pH 7.5 containing 1.6% albumin until the completion of epiboly (Guralp et al. 2016). Ringer's solution was replaced every 2 h. After completion of epiboly, embryos were transferred to a second culture solution (1.8 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing 0.01% penicillin and 0.01% streptomycin and incubated at 15°C. A control group of approximately 80 embryos with chorion was incubated in filtered and aerated tap water in 120 mm plastic Petri dishes with water replaced every 4 h.

Definition of stages. The developmental stages were defined morphologically by stereomicroscopy (Leica M165FC; Leica, Germany) and photographed (Leica DFC425C). The embryos were observed during the somitogenesis period every 2 h.

Observation of PGCs. Zebrafish *nos3* 3'UTR mRNA containing green fluorescent protein (GFP) was synthesized using the mMESSAGE mMACHINE Kit (Life Technologies Corp., USA) according to manufacturer's instructions, and 300 ng/µl in 0.2M KCl was injected into the blastodisc of 2-cell pikeperch embryos to visualize the PGCs (Saito et al. 2006) (Figure 1A). PGCs were observed in darkness from the 100% epiboly until hatching using fluorescence stereomicroscopy (Leica M165FC), and photographed (Leica DFC425C).

Tracking migrating PGCs. The migrating PGCs were recorded by a stereomicroscope equipped with a camera (Leica DFC425C) for 2-hour periods with 30 s between frames. The position of each PGC was marked, and photos were merged into a single image in order to track PGCs.

Blastomere transplantation. The donor embryos were labelled with GFP-*nos3* 3'UTR mRNA and tetramethylrhodamine (TMR)-dextran (molecular weight (MW) = 10 000) at the 2–4 cell stage. The BT from labelled into non-labelled embryos was performed as described by Saito et al. (2010), so that transplanted PGCs and somatic cells could be visualized in the chimeric embryos. We aspirated the labelled blastomeres into a glass microneedle connected to a microinjector (Eppendorf, Germany) from the marginal region of the donor embryo blastoderm at the blastula stage and transplanted them into the same region of the host blastoderm at the same stage (Figure 3A). The chimeric embryos were examined under a fluorescence stereomicroscope (Leica M165FC). Images of the embryos were obtained using the appropriate filters for GFP and rhodamine fluorescence and were merged into a single image using ImageJ software.

RESULTS

Late embryonic development in pikeperch. The embryonic development in pikeperch was separated into cleavage (1.5–7.5 hpf), blastula (9–18.75 hpf), gastrula (21–39 hpf), segmentation (45–105 hpf), and hatching (125 hpf) periods based on morphological features. Three groups of embryos from different females were observed, and the embryonic periods were completed by hatching period at 125 hpf.

PGC migration in pikeperch. PGCs were observed in all embryos injected with synthesized GFP-*nos3* 3'UTR mRNA from 100% epiboly at 39 hpf. The mean number of PGCs in each embryo at the early segmentation period was 12.6 (standard deviation 8.52, range = 2–35). The PGCs showed migration to, and localization at, the gonad area during development and were confirmed as PGCs.

Time-lapse imaging and analysis revealed the migration pattern of PGCs from the time and place of their appearance to localization at the gonadal ridge (Figure 1). The PGC migration patterns and localization were similar among embryos although in some the PGCs appeared only, or in lower numbers, on one side of the body. At the beginning of the segmentation period (the 1–12

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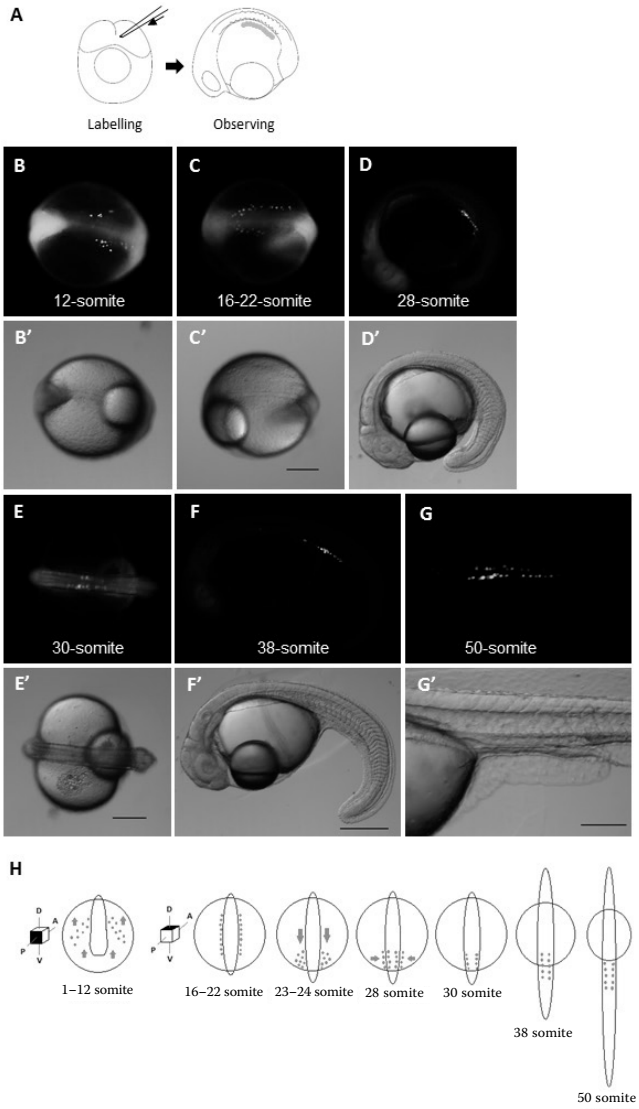

Figure 1. Migration pathway of primordial gem cells (PGCs) in pikeperch embryos at 15°C
(**A**) schematized experimental design of PGCs observation, PGCs were labelled with green fluorescent protein (GFP)-*nos*3 3'UTR mRNA; (**B–B'**) PGCs migrated from posterior to anterior part of embryonic body at 12 somite stage (54 hpf); (**C–C'**) PGCs localized at ventral side of embryonic body at 16–22 somite stage (64 hpf); (**D–D'**) PGCs migrated to posterior at 24–28 somites (70 hpf); (**E–E'**) PGCs localized on both sides of germinal line on ventral side of embryonic body at 30 somites (78 hpf); (**F–F')** PGCs extended through yolk to germinal line (90 hpf); (**G–G'**) PGCs localized on both sides of germinal line (125 hpf); (**H**) scheme of PGC migration, posterior view at 1–12 somites and dorsal view at 16–50 somites. Scale = 200 μm

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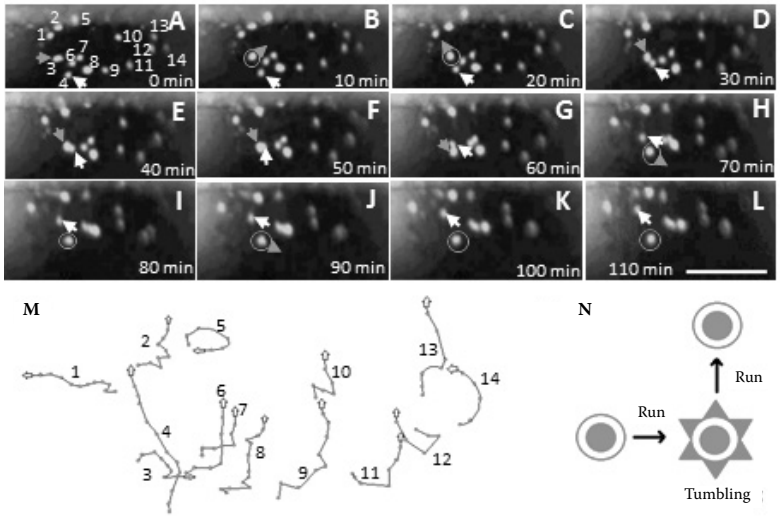

Figure 2. Primordial gem cells (PGCs) migration at 28 somites for 110 min (**A**–**L**) in the left cluster of the PGCs, 14 migrating PGCs are clearly observable. Two migrating PGCs (3 and 4) are indicated by white and red arrows intercepting. During migration, the PGCs showed tumbling behaviour. The tumbling is indicated by red circles with arrowheads showing the direction of movement. A single arrow indicates the migration orientation and position of PGC. A single circle shows position of PGC; (**M**) = migration pathway of each PGC (1 to 14); (**N**) = illustration of migrating PGCs behaviour. PGCs are in green and the direction of movement is shown by red arrows. Tumbling movement is illustrated in all possible orientations by red arrows on the circle. Scale = 100 μm

somite stage), the PGCs were widely distributed on both sides of the trunk in the posterior half of the embryonic body; some were located ventrally, especially in the posterior near the developing tail bud (Figure 1B). As the trunk and tail bud became more prominent around the 16–22 somite stage (Figure 1C), the PGCs began to migrate and were loosely aligned along both sides of the developing somites. The PGCs on the posterior ventral surface of the body migrated laterally and joined to form a loose PGC alignment. The PGCs in the anterior region migrated posteriorly and narrowed their distribution area. During this shift, the width of the PGC distribution increased. The aligned posterior PGCs continued to migrate but they were confined to target areas. At the 23–24 somite stage, PGCs continued to migrate towards the axis of the body and a more confined area, to form two PGC clusters. The subsequent step, at the 28 somite stage, represented completion of active migration, positioned on the ventral side of the embryonic body (Figure 1D). The PGCs displayed slower movement for a short distance on the ventral side of the body during the late segmentation period until located at the origin of the germinal line at the end of the yolk extension. The final step of migration, at the 30–50 somite stage, was the localization of PGCs in alignment on both sides of the germinal region of the embryo (Figure 1E–H).

During the migration process, PGCs exhibited active behaviour as reported in zebrafish PGC migration, characterized by the formation of protrusions and "run and tumble" behaviour (Figure 2). Each PGC moved through the pathway and, after tumbling, either continued to move in the same direction or in other directions for varying distances depending on the its original position.

Blastomere transplantation in pikeperch. Twelve BT chimeras were produced, and eight survived to hatching. All showed donor-derived cells

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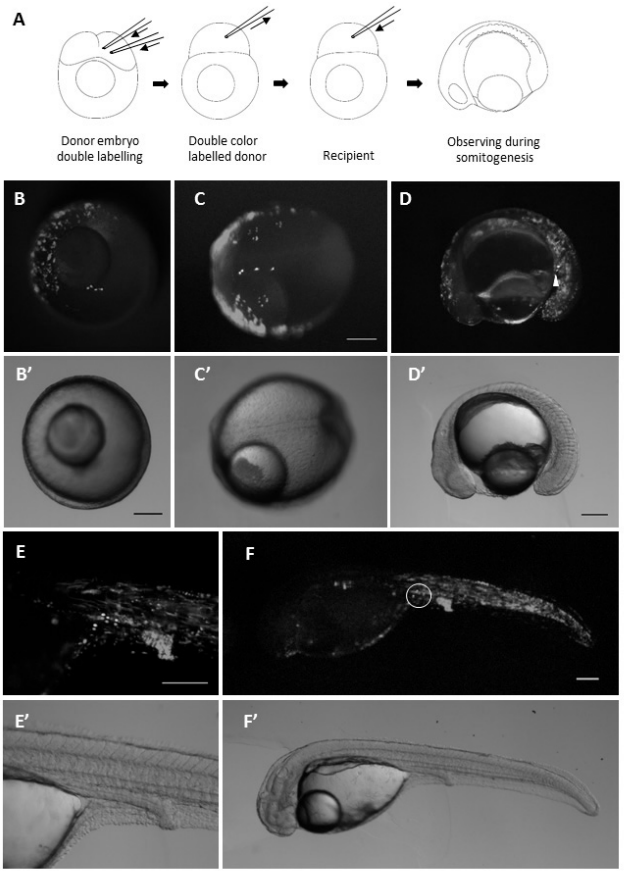

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	Total	Survive	PGSs positive	PGSs negative
RNA-injected	42	41	41	0
BT-chimera	12	8	2	6

Figure 3. Blastomere transplantation (BT)

(**A**) schematic illustration of BT experimental design. The donor embryos were co-labelled with green fluorescent protein (GFP)-*nos3* 3'UTR mRNA and tetramethylrhodamine-dextran at 1–2 cell stage. Labelled donor blastomeres were aspirated into a glass needle and injected into the recipient at the blastula stage. Transplanted donor cells were observed during somitogenesis; (**B–F**) chimeras under light and fluorescence microscopy – red-labelled cells are somatic cells, and GFP-labelled cells are primordial gem cells (PGCs) (**B–B'**) at 4 somite stage; (**C–C'**) at 16 somite stage; (**D–D'**) at 22 somite stage, two PGCs indicated by arrowhead; (**E–E'**, **F–F'**) at 44 somite stage embryos, PGCs indicated by circle; (**G**) = efficacy of BT. Scale = 200 μm

in the developing body. As the chimeric embryos developed, donor cells mingled with host cells and were distributed throughout the body, including somites, brain, and intestine (Figure 3B–F). Two of the eight embryos exhibited GFP-labelled PGCs at the gonadal ridge (Figure 3B–F).

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DISCUSSION

PGC migration in pikeperch. The migration route of PGCs was identified by GFP visualization for the first time in pikeperch. The efficacy of microinjection has been evaluated by the presence of PGCs in embryos of 100%, as reported in ice goby, zebrafish, and Pacific herring (Saito et al. 2006). The appearance of PGCs on only one side of the body in some embryos was similar to findings in these studied model fish species. We observed 2–35 labelled PGCs in pikeperch embryos, similar to reports in other species (Saito et al. 2006). The number of visualized PGCs in the embryos can depend on the efficacy of microinjection, and may not show the actual quantity of PGCs. Saito et al. (2011) reported that the number of GFP-positive PGCs varied among eel embryos, due to the difficulty of microinjection. PGCs have been previously visualized from 100% epiboly in tench; from 50% epiboly in zebrafish, medaka, loach, and eel; and from 90% epiboly in ice goby (Saito et al. 2006, 2011). Our results were consistent with studies regarding the conserved function of GFP-*nos3* 3'UTR mRNA in teleosts (Saito et al. 2006).

We observed that the PGCs in pikeperch migrated to the region of gonad development and were localized there, consistent with the results of studies in other fish species (Saito et al. 2004, 2006; Li et al. 2015; Wang et al. 2015). Studies have revealed differing migratory patterns of PGCs during embryonic development among fish species. The migration path of PGCs in pikeperch showed similarities with goldfish; however, unlike goldfish and similar to herring, the PGCs in pikeperch clustered in the posterior region of the body. A comparison of our results with other reports shows wide variations in PGC migratory paths among species. The source of the differing migration patterns among fish may be the combination and balance of chemo-attractant signals, which have been described in detail for zebrafish and medaka (Doitsidou et al. 2002; Boldajipour et al. 2008; Saito et al. 2015). In olive flounder, *sdf-1* mRNA encoding a protein that guides PGC migration is expressed in the ventral region of the embryonic body at the 15 somite stage, and PGCs are localized in this region at that stage. In zebrafish, *sdf-1* is not expressed in this region, and PGCs do not migrate toward the ventral side of the body at the 15 somite stage. These species use the same attractant system, but PGC migration shows different patterns.

Blastomere transplantation. We successfully established a technique to produce germ-line chimeras in pikeperch with the rate of efficacy of PGC transfer in chimeras higher than reported in zebrafish (Saito et al. 2010). While donor-derived PGCs were observed in the gonad region in 8.97% of zebrafish chimeras, 25% of pikeperch chimeras exhibited donor PGCs at the gonad region in our study. The donor cells were well distributed throughout the blastoderm, and the distribution of the donor somatic cells in pikeperch BT chimeras was similar to that of zebrafish (Saito et al. 2010) and rainbow trout *Oncorhynchus mykiss* (Takeuchi et al. 2001). Transplanted somatic cells were evenly distributed throughout the embryonic body including somites, brain, and intestine, while PGCs were located in the gonad region.

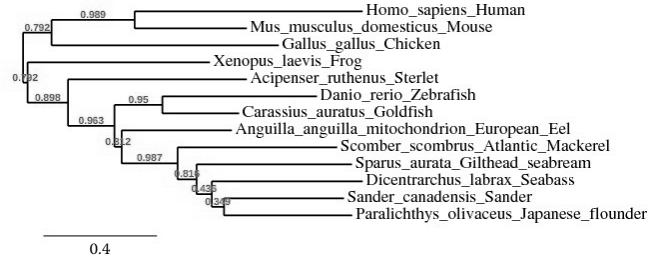

Figure 4. Phylogenetic relationships of the *Sander* species to other eukaryotes
Phylogenetic tree with branch length is proportional to the number of substitutions per site. It shows the closest marine fish species, which can potentially be donor species for blastomere transplantation in percids. Cytochrome b sequence of species was used to construct the tree using Phylogeny.fr software after searching in NCBI

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It has been reported that the phylogenetic relationship does not affect the migration of donor PGCs into the gonad region of the host in interspecies transplantation (Saito et al. 2010). On the other hand, donor-derived PGCs did not develop in the chimeras generated by a single PGC transplantation between distantly related family members, although the donor-derived germ cells did migrate and localize to the genital ridge of recipient embryo (Saito et al. 2011). Therefore, BT might be effective in generation of inter-species germ-line chimeras between phylogenetically close family members, considering the phylogenetic position of pikeperch and its developmental pattern, which is similar to marine fish such as sea bass *Dicentrarchus labrax* and Japanese flounder *Paralichthys olivaceus* (Figure 4). Pikeperch can be a potential host to generate inter-species germ-line chimeras between freshwater and marine species. Micromanipulation will allow surrogate production of endangered and commercially valuable percids, for instance, asprete *Romanichthys valsanicola*, and, potentially, reproduction of brackish water species such as European perch *Perca fluviatilis* and marine fish such as sea bass and Japanese flounder in freshwater.

CONCLUSION

In this study, we described the migration of PGC, based on the knowledge of the late embryonic development of pikeperch from gastrula to hatching. We established a reliable technique to transplant PGCs in pikeperch embryos. Further study is needed to determine whether donor germ cells in the chimera will differentiate into functional gametes. Deletion of endogenous PGCs must be established to conduct exclusive production of donor gametes in pikeperch. More importantly, although it is often overlooked, a method of culturing a small number of manipulated larvae and juveniles is needed for practical surrogate production. Based on the described techniques and results of the present study, GCTT using a pikeperch model can be realized.

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CHAPTER 5

GENERAL DISCUSSION

Preparation of embryos

Studies focusing on broodstock management in RAS, provided large numbers of pikeperch eggs from which to choose (200000 kg^{-1} body weight) and sperm (30.109.L^{-1} concentration) (Blecha et al., 2015; Schlumberger and Proteau, 1996; Zakęś and Demska-Zakęś, 2009). The transparency of the pikeperch embryos is an advantage for developmental biological studies; however, egg stickiness and hard chorion presented some difficulties for micromanipulation studies. I had to remove the stickiness and the chorion to be able to perform embryo manipulation (Güralp et al., 2016, 2017). The elimination of egg stickiness is also a crucial step to incubate large numbers of eggs in Weiss-Zuger jars. The most widely used treatments are proteolytic enzymes such as alcalase or tannic acid (Kristan et al., 2015; Żarski et al., 2015). Tannic acid treatment is applied at 30 min after fertilization with 0.75 g.L^{-1} for 1–2 mins (Żarski et al., 2015). In our RAS, alcalase has been used efficiently at the concentration of 1.5–2 ml.L^{-1} for 2 min just after insemination (Kristan et al., 2015). We did not use tannic acid treatment because the treatment needs to wait 30 minutes after fertilization, and also tannic acid makes the chorion very hard (Żarski et al., 2015) making chorion more difficult to remove. Conversely alcalase treatment softens the chorion, and was not useful to remove chorion because surface tension of the membrane increased. Other enzymatic treatment including a trypsin and urea combination in an isotonic solution was successful for dechorionation for micromanipulation studies in different fish species such as goldfish (Otani et al., 2002; Yamaha et al., 1986), zebrafish (Lin et al., 1992; Saito et al., 2008), loach (Nakagawa et al., 2002)germline chimeras using the loach (Misgurnus anguillicaudatus, and tench (Linhartova et al., 2014). The concentration of trypsin, urea and Ringer's solution varies among species; in goldfish and loach as 0.1% Trypsin (Difco) and 0.4% urea in Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) (Nakagawa et al., 2002; Otani et al., 2002), tench as 0.1% trypsin and 0.4% urea in Ringer's solution (112.2 mM NaCl, 3.35 mM KCl, 2.27 mM $CaCl_2.2H_2O$, 23.81 mM NaHCO3) (Linhartova et al., 2014), zebrafish as 0.1% trypsin, 0.002% actinase E (Kaken), and 0.4% urea in Ringer's solution (128 mM NaCl, 2.8 mM KCl, and 1.8 mM $CaCl_2$) (Saito et al., 2008). In the trypsin solution, pH must be 8 to 9 to digest chorion. In zebrafish and goldfish trypsin/urea in Ringer's solution completely digests the chorion without additional mechanical treatment. In our study on pikeperch, we tested several different concentration, of trypsin/urea and found 0.2% trypsin and 0.4% urea removed the stickiness of the eggs and made the chorion soft enough to remove by fine forceps (Güralp et al., 2016). Although the dechorionation of small (919.4 μm) pikeperch embryos individually was challenging, it was possible to observe embryo development in high resolution without chorion and to apply micromanipulation techniques in pikeperch embryos (Güralp et al., 2016).

Embryo development

In our study, techniques to obtain fertilized eggs and removal of chorion allowed to study embryo development in detail. We used the similar terminology with other researchers to describe the morphology and characterize the stages (Alix et al., 2015; Arakawa et al., 1999; Cooper, 1978; Cucchi et al., 2012; Fujimoto et al., 2004, 2006; Iwamatsu, 2004; Kimmel et al., 1995; Kováč, 1994, 2000; Mansueti, 1964; McElman and Balon, 1979; Paine and Balon, 1984a, 1984b; Tsai et al., 2013).

One of the main differences in embryo development among fishes is the structure of the eggs. Some fish have oil globules in the eggs (Alix et al., 2015; Bermudes and Ritar, 1999;

Cooper, 1978; Cucchi et al., 2012; Kováč, 1994, 2000; Mansueti, 1964; McElman and Balon, 1979; Paine and Balon, 1984a, 1984b). The change in oil droplet position effects rotation of the embryo during development. This has been described in walleye Stizostedion vitreum and the northern logperch *Percina caprodes semifasciata*, which have oil droplet movement associated with cell migration during gastrulation (McElman and Balon, 1979; Paine and Balon, 1984b). In addition, pikeperch have one of the smallest fertilized egg diameter (0.9–1.0 mm) (Güralp et al., 2016) among percid species. These structural features hamper observing, photographing and manipulating the embryo. Although we placed the embryo in a suitable position size of the whole on an agar plate, the embryo slowly rotated until the vegetal side was upmost.

There were differences in the gastrulation period of pikeperch reported for other teleosts. Formation of the germ ring and the embryonic shield occurred at 40% and 50% epiboly, respectively, in pikeperch. In zebrafish, goldfish, and loach, germ ring, and embryonic shield are seen at 50% epiboly (Fujimoto et al., 2006; Kimmel et al., 1995; Tsai et al., 2013). The duration of the gastrula period among percids ranges from 6 hours in rainbow darter *E. caeruleum* to 23 hours in Danube streber *Z. streber* (Kováč, 2000; Paine and Balon, 1984a). Gastrula in pikeperch was 24 hours, not previously reported in percids, and shows the closest similarity to Danube streber with 23 hours, and total duration of embryo development of Danube streber is 24 days (Kováč, 2000). The chronology of embryo pattern and organ development was similar to that in other investigated percids (Schaerlinger and Żarski, 2015), except yellow perch *P. flavescens* and rainbow darter, in which the mouth opening was reported to occur before hatching, during segmentation (Mansueti, 1964; Paine and Balon, 1984a). Although the duration of embryo development from fertilization to segmentation of pikeperch is similar to that of *Z. streber* and *P. flavescens*, segmentation duration, the time to hatching, and duration of hatching varies. *Danube streber* develops in a chorion for 14 days and exists as a free embryo for 10 days (Kováč, 2000), but yellow perch hatches at 26 dpf and exists as a free embryo for three days (Mansueti, 1964) These differences are thought to be associated with yolk sac consumption and spawning mode (pelagic or demersal) (Schaerlinger and Żarski, 2015).

The total duration of embryo development is different (10.2 days from fertilization to first feeding) between species. Pikeperch is one of the species that has the shortest embryonic periods among percids, which range from 8 to 30 days (Alix et al., 2015; Cooper, 1978; Cucchi et al., 2012; Kováč, 1994, 2000; Mansueti, 1964; McElman and Balon, 1979; Paine and Balon, 1984a,b). It can be an advantage to carry out experiments in a relatively short period so that pikeperch can be a potential model percids species with flexible incubation temperatures and relatively short embryo development.

The importance of the temperature effect on developmental rate

Temperature is one of the most influential environmental factors on fish development. The range of optimal temperature is species-specific (Feiner and Höök, 2015).The optimal incubation temperature for pikeperch embryos has been reported as 15 °C (Schlumberger and Proteau, 1996). Moreover, pikeperch grows in relatively short incubation period under optimal conditions at 65–110 day-degrees (d°) that makes pikeperch a good potential candidate as a laboratory model for percids. In some experiment, however, such as embryonic cells transplantation, the speed of embryo development is slowed by incubating at lower temperatures. For this purpose, we examined the viable temperature range of embryos at each stage to avoid an accidental death.

The developmental rate of pikeperch embryos was slower at 10 °C than 15 °C. The embryos incubated at 10 °C reached to 1k-cell stage at 17.5 hours post-fertilization (hpf) that delayed 5.5 hours compared to embryos incubated at 15 °C. The fertilization rate at 10 °C was significantly lower than that of 15 °C. The optimal temperatures for pikeperch fertilization has been reported as 12–16 °C and our results were consistent with the reports (Feiner and Höök, 2015; Schlumberger and Proteau, 1996). However, there were no significant difference in survival rate between the groups fertilized and incubated at 10 °C, and groups fertilized at 15 °C and incubated at 10 °C. The group that fertilized at 15 °C and then incubated at 10 °C develop normally as controls. Therefore, by fertilizing eggs at 15 °C and incubating them at 10 °C, embryos can be cultured and hatched safely without loosing their fertiliation capacity (Chapter 2). This information is beneficial especially for transplantation experiment. By incubating the embryos at 10 °C, it is possible to shift and extend the time of the transplantation experiment that was proposed before (Güralp et al., 2016; Yamaha et al., 2003).

The important embryonic events and the optimum stage for micromanipulation

Our research described detailed developmental events during the blastula stage of the pikeperch with investigation of yolk syncytial layer (YSL) formation and mid-blastula transition (MBT) (Güralp et al., 2016). During embryo development, the number of blastomeres synchronously increase as described in model fish (Fujimoto et al., 2004; Iwamatsu, 2004; Kane and Kimmel, 1993; Yamaha et al., 1999). We observed the synchronous cleavage pattern in pikeperch embryos until 13.5 hpf in 1k-cell stage at 15 °C. Synchronized cell cycles were repeated ten times, identical with loach and medaka embryos (Fujimoto et al., 2004; Iwamatsu, 1994; Kane and Kimmel, 1993) but unlike goldfish with 9 synchronous cell cycles (Yamaha et al., 1999) and zebrafish with 8 synchronous cycles continues during 9th cycle (Kane and Kimmel, 1993). Zygotic gene expression and active cell migration began at MBT in *Xenopus* and zebrafish (Kane and Kimmel, 1993; Newport and Kirschner, 1982). In pikeperch, asynchronous cell division began after 13.5 hpf (after 1k-cell stage) at 15 °C, so this stage is probably the MBT in pikeperch. In addition, the blastodisc isolation assay demonstrates that embryos have the high competency for manipulation at 1k-cell stage compared to before the 512-cell stage and after 14 hpf. Moreover, we observed that the marginal blastomeres broke down during the 512- to 1k-cell stages and thereafter the nuclei from these blastomeres formed YSL that is identical to that of zebrafish (Kimmel and Law, 1985). According to reports of zebrafish and goldfish, the yolk mass with YSL signals that induce the mesoendoderm and the dorsal axis to the pluripotent blastodisc after the mid-blastula stage (Chen and Kimelman, 2000; Yamaha et al., 1998; Mizuno et al., 1996). In addition, it is reported that YSL plays a central role in patterning of germ layer and morphogenesis in fishes that makes it a convenient indicator for staging (Kimmel et al., 1995). These findings concurred with the fact of that remaining blastomeres at the animal pole region retained a pluripotency until the late blastula stage (Ho and Kimmel, 1993; Yamaha et al., 1998). The tolerance to the blastula isolation assay seemed to be related to the size of blastomeres. Although blastomeres at the 256-cell stage have pluripotency, survival rate was low after performing blastodisc isolation at this stage. Probably, blastomeres are fragile and easily damaged by the manipulation with the glass needle but as blastomeres become smaller through division, they develop more tolerance to surgical manipulation. On the other hand, survival rate of embryos declined after blastodisc separation at 15 hpf. At this stage and thereafter, the embryos showed sphere-shaped with high surface tension. It is reasonable to think that the high surface tension on the embryo prevents the restoration of blastodisc. Saito et al. (2011) described that blastomeres transplantation

can be a useful technique to transfer PGCs. Transplanted blastomeres and PGCs are capable of contributing to the development of the recipient without mismatches of tissue affinity that can cause deformation of the embryo, when the transplantation is performed between same or closely related species. We found that such manipulations were also applicable to pikeperch embryos, although they have limited tolerance to micromanipulation; the optimal period for micromanipulation in pikeperch embryos is 12–14 hpf at 15 ºC. This information was successfully used for PGCs transplantation experiment for pikeperch as shown below (Güralp et al., 2016).

PGC migration

We observed PGCs with green fluorescent protein (GFP) labeling technique for the first time in pikeperch. The success of microinjection for visualization of PGCs was 100% as reported in ice goby, zebrafish, and Pacific herring (Saito et al., 2006). In our study, the first appearance of GFP-positive PGCs under the fluorescence stereomicroscope was at 100% epiboly which was same with the study in tench (Linhartova et al., 2014). Occasionally, we found embryos with GFP-labelled PGCs only on one side of the embryonic body. Probably it was caused by partial labeling of a blastodisc due to high molecular weight of the injected mRNA as discussed in Saito et al. (2006). The number of GFP-labelled PGCs had a range from 2 to 35. The difficulty of microinjection procedure might affect the number of visualized PGCs. Saito et al. (2011) have also reported the wide range of numbers of GFP-labelled PGCs in eel embryos, and they mentioned that it was difficult to inject mRNA into eel eggs due to the embryonic rotation by an oil droplet. Although the average number of PGCs obtained in this research might be varied by increasing the concentration of mRNA and a performance of a microscope, I was able to estimate the migratory path of pikeperch PGCs during development. A comparison of our results with other reports showed wide variations in PGC migratory paths among species (Saito et al., 2006). The migration pathway of the PGCs is not identical among fish (Linhartova et al., 2014; Saito et al., 2006, 2010, 2011, 2014; Wang et al., 2015), although we found similarities with goldfish. For example, the PGCs in pikeperch clustered in the posterior region of the body after alignment along both sides of the developing somites as illustrated in goldfish (Saito et al., 2006). The variation of PGC migration among fish could be explained by the differences of temporal and spatial expression pattern of PGCs guidance signals (*cxcr4/ sdf1* chemokine system) (Boldajipour et al., 2008; Doitsidou et al., 2002). Also, different species have different physiology and proportion of the cells with the activity of *sdf1* and *cxcr7* in tissues surrounding the migrating PGCs (Mahabaleshwar et al., 2008). Even though species use the same attractant system, PGC migration have different patterns. For example, Wang et al. (2015) showed that the expression of *sdf-1* mRNA encoding a protein and the localization of PGCs were simultaneous at the 15-somite stage in the ventral region of the embryonic body of olive flounder. However, *sdf1* does not express in the ventral region of the embryonic body of zebrafish, and PGCs do not migrate to the place. In pikeperch embryos, we found that the PGCs migrated to the ventral region of embryonic body as seen in olive flounder during the somitogenesis period. The information of PGCs location at each stage in pikeperch embryo is useful to evaluate the potential and efficiency of blastomere transplantation (BT).

Blastomere transplantation

A germline chimera can be generated by transplanting PGCs from a donor embryo to a host. However, a great deal of knowledge about the embryo and PGCs development in the target fish is needed to perform an efficient transplantation. As we know, the PGCs localize around the peripheral region of the blastoderm at blastulation (Yoon et al., 1997). As described by Lin et al. (1992), we randomly aspirated blastomeres into a micro-glass needle from a donor pikeperch embryos and transplanted into a host pikeperch embryo at the same stage. We observed the GFP labelled PGCs of donor embryo in the chimeric embryo. The ratio of the correctly localized donor-derived PGCs in the gonad region of the host was a good indicator of the BT efficiency. We observed 25% of pikeperch chimeras had donor-derived PGCs which migrated to the gonadal ridge while Saito et al. (2010) reported 8.97% in zebrafish chimeras. We observed that the other donor-derived tetramethylrhodamine dextran-labelled cells except PGCs, were well distributed throughout the blastoderm and the embryonic body, while PGCs were located in the gonad region as described in other fish (Saito et al., 2010; Takeuchi et al., 2001).

Blastomere transplantation, including PGCs can be efficient between same or closely related species. Donor blastomeres well mingled with host cells during embryo development, and migration of the donor PGCs is not affected by the chimerism. However, in the case of chimeric combination between distantly related species, donor derived somatic cells disturb the embryo development by forming aggregations. These aggregations induce abnormality and inhibit PGCs migration (Saito et al., 2010). Probably, the marginal somatic blastomeres, which were transplanted with PGCs, express guidance signals, *sdf-1a*, in the chimera, and the aggregation of these cells misguide the PGCs. In fact, the germ-line chimera could be induced by transplanting an isolated PGC without somatic cells (Saito et al., 2008). On the other hand, in the case between distantly related species, although the donor-derived single PGC could migrate and localize in the gonadal ridge of the host embryo, the donor PGCs disappeared during gonad development in the chimeras (Saito et al., 2011). Thus, the surrogate production technology might be functional only between close related species in fish.

As I describe in chapter 4, there are many closely related species with pikeperch, both in freashwater and in seawater, and many of them show similar embryonic developmental patterns. Therefore, we propose that BT or single PGC transplantation might be efficient in the generation of inter-species germline chimeras. Pikeperch can be a potential hosts or donors to generate inter-species germline chimeras between freshwater and marine species after more data accumulation of other potential species such as endangered asprete *Romanichthys valsanicola*, and commercially valuable European perch *Perca fluviatilis*, olive flounder *P. olivaceus* and sea bass *D. labrax*.

Conclusions

In this thesis, we describe several specific methods which are required for sustainable reproduction of fish with the new biotechnological techniques, or so-called surrogate production. The main results in this thesis could be informative and useful for generation of germline chimera using pikeperch embryos.

So far, transplantation of blastomeres (BT) containing PGCs is the only available method for the generation of a germline chimera in pikeperch. In addition, BT will be a useful tool to investigate the function of genes in pikeperch as a proposed model fish in percids.

A germline chimera can be generated through spermatogonial transplantation into larvae (Lacerda et al., 2010). However, pikeperch larvae were sensitive and did not survive after

transplantation, or even mild anaesthesia (Güralp, unpublished data). It will be a task for further research to find a more effective method to allow germ cell transplantation in different stages of developing pikeperch. A possible variant could be the use of different anaesthesia treatments at different temperatures with variants of durations.

Moreover, further study is needed to determine whether donor germ cells in the chimera will differentiate into functional gametes. For this goal, deletion of endogenous PGCs with an effective sterilisation method must be established to conduct an exclusive production of donor gametes in pikeperch.

Another issue that needs to be addressed is the preservation and supply of sustainable germ cells by cryopreservation or *in vitro* cultivation of gonadal stem cells. We have already performed a preliminary experiment on spermatogonia isolation by Percoll gradient method in pikeperch (Güngör, 2015). It will be a task to improve this or other germ-cell isolation techniques in pikeperch.

Although it is often overlooked, it will be necessary to improve the special care of a small number of manipulated larvae and juveniles in captivity that will be needed for practical surrogate production.

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ENGLISH SUMMARY

Embryo development and transplantation of primordial germ cells in pikeperch *Sander lucioperca*

It was the purpose of this thesis to implement primordial germ cell (PGC) transplantation in pikeperch, one of the new biotechnological reproductive methods, and for this to explain the necessary details about embryo development and PGC migration. We provide several specific methods such as GFP labeling and blastodisc surgery which are required for efficient assessment of the transplantation technique. The main thesis output could be useful informative for generation of germline chimera of pikeperch.

In the first study, we described detailed pikeperch embryo development at 15 ºC to first feeding and demonstrated effects of temperature on the rate of embryogenesis to determine temperature limits for slowing development with minimum negative effects on growth and survival rate. Embryonic developmental stages incubated at 15 ºC were identified as zygote, 0–1.5 hour post- fertilization (hpf); cleavage, 2.5–7.5 hpf; blastula, 9–18.75 hpf; gastrula, 21–39, hpf; segmentation, 45–105 hpf; and hatching, 125–197 hpf. We also developed a technique to soften the pikeperch chorion by treating with 0.2% trypsin and 0.4% urea in Ringer's solution in order to remove it by forceps for in-depth observation. Additional groups of eggs were fertilized and incubated at different temperatures to document embryo developmental stages, developmental rate, and survival. The optimum fertilization and incubation temperature was 15 ºC, with the highest fertilization, survival, and hatching rates. Embryo development was drastically slowed at 10 ºC, with 45% of fertilized embryos surviving to hatching. Development was accelerated at 20 ºC, with a 56% survival rate of fertilized embryos. At 25 ºC, embryos did not develop to the blastula stage. After the series of experiments to characterize the embryo development of pikeperch, we concluded that pikeperch could be a valuable percid model for research in which flexible incubation temperatures is required.

In a second study, we described the important early embryonic events, namely, yolk syncytial layer (YSL) formation and midblastula transition (MBT) during the blastula stage in pikeperch embryos. The chorion was removed as we described in the first study. The YSL was formed after the breakdown of marginal cells during the 512- to 1k-cell stages. Cell division analysis by 4′-6-diaminido-2-phenylindole (DAPI) staining revealed that transition from synchronous to asynchronous division occurred after the 10th cleavage (1k-cell stage). Our results indicate that MBT starts after this stage. Next, we performed blastodisc isolation assays to find the competent stage for embryonic manipulation. Embryos were manipulated by using a microneedle every hour from the 512-cell to the sphere stage, and then developmental rates were evaluated at the hatching stage. The highest survival rate was obtained when we performed this manipulation at the 1k-cell stage. These results clearly showed that the MBT is the best stage for transplantation of PGCs or any cells in pikeperch.

In the third study, we described PGC migration and performed blastomere transplantation in pikeperch. PGCs were visualized by injection of synthesized green fluorescent protein (GFP) within the 3′untranslated region (UTR) mRNA of *nanos3*. GFP-positive PGCs appeared in all embryos at approximately 100% epiboly. Time-lapse imaging revealed the PGC migration pattern from their initial appearance to the location at the gonadal ridge. We conducted blastomere transplantation at the blastula stage. Donor embryos were labeled with GFP-*nos3* 3′UTR mRNA and tetramethylrhodamine dextran to differentiate PGCs and somatic cells, respectively. Twelve blastomere transplantation chimeras were produced, with eight survived to hatching. All exhibited donor-derived somatic cells in the developing body. The PGCs from donor embryos were observed to migrate towards the gonad region of the host embryos. Our results indicated that blastomere transplantation can be successfully applied in pikeperch, and these findings may be useful to produce germline chimeras in percids.

CZECH SUMMARY

Embryonální vývoj a transplantace primordiálních zárodečných buněk u candáta obecného *Sander lucioperca*

Cílem této práce bylo popsat embryonální vývoj a migraci primordiálních zárodečných buněk neboli primordiálních gonocytů (PGC) a realizovat transplantaci PGC u candáta obecného. V práci popisuji několik specifických metod, jako je GFP značení a mikromanipulace s blastodiskem, které jsou nutné pro posouzení účinnosti transplantační techniky. Hlavní výsledky této práce mohou být využity pro vytvoření chimér zárodečné linie candáta obecného.

V první studii jsme detailně popsali embryonální vývoj candáta obecného až do prvního krmení při 15 °C a efekt teploty na rychlost embryogeneze při stanovení teplotních limitů pro zpomalení vývoje, a to s minimálními negativními důsledky na růst a míru přežití. Vývojová stadia embryí inkubovaných v 15 °C byly identifikovány jako zygota, 0–1,5 hodiny po oplození (hpf); rýhování, 2,5–7,5 hpf; blastula, 9–18,75 hpf; gastrula, 21–39 hpf; segmentace, 45–105 hpf; a kulení, 125–197 hpf. Také jsme vyvinuli techniku změkčení chorionu candáta obecného díky použití 0,2% trypsinu a 0,4% močoviny v Ringerově roztoku za účelem snadného odstranění chorionu pomocí pinzet, což umožňuje detailnější pozorování. Navíc byla skupina jiker oplozena a inkubována v rozdílných teplotách za účelem zdokumentování vývojových stadií embrya, rychlosti vývoje a míry přežití. Optimální inkubační teplota, s nejvyšším oplozením, mírou přežití a mírou vykulení, byla 15 °C. Embryonální vývoj byl významně zpomalen při 10 °C, s 45 % vykulených embryí (počítáno z oplozených jiker). Vývoj byl zrychlen při 20 °C, s 56% mírou přežití oplozených embryí. Při 25 °C embrya nedosáhla ani stadia blastuly. Po sérii experimentů, charakterizujících embryonální vývoj candáta obecného, jsme dospěli k závěru, že pro výzkum, kde jsou požadovány flexibilní inkubační teploty, může být candát obecný z čeledi okounovitých hodnotným modelem.

V druhé studii jsme popsali důležité fáze časné embryogeneze, konkrétně formování žloutkové syncytiální vrstvy (YSL) a přechod midblastuly (MBT) během stadia blastuly embryí candáta. Chorion byl odstraněn způsobem popsaným v první studii. YSL se formovalo po rozpadu marginálních buněk během stadia 512–1000 buněk. Následná analýza buněčného dělení pomocí barvení 4′-6′-diaminido-2-phenylindole (DAPI) ukázala, že přechod ze synchronního k asynchronnímu dělení se vyskytuje po desátém rýhování (stadium 1000 buněk). Naše výsledky naznačují, že fáze MBT nastává až po tomto stadiu. Dále jsme provedli test isolace blastodisku, za účelem nalezení kompetentního stádia pro následnou embryonální manipulaci. S embryi bylo manipulováno za použití mikrojehly a mikromanipulátoru. Každou hodinu od stadia 512 buněk do stadia „sphere stage". Poté byly vyhodnoceny vývojové rychlosti ve stadiu kulení. Nejvyšší míra přežití byla zjištěna při provedení manipulace ve stadiu 1 000 buněk. Výsledky jasně ukazují, že embryo candáta obecného ve fázi MBT je nejvhodnější pro transplantaci PGC nebo jinou manipulaci.

Ve třetí studii jsme popsali migraci PGC a provedli jsme transplantaci blastomer candáta obecného. PGC byly vizualizovány díky injikaci uměle syntetizované mRNA GFP-nos3 3′UTR. GFP-positivní PGC se objevily ve všech embryích ve stadiu přibližně 100% epiboly. Pomocí časosběrného snímání jsme popsali migrační vzor PGC od jejich počátečního objevení až po lokaci v zárodečné rýze. Během stadia blastuly jsme provedli transplantaci blastomer. Embrya donora byla značena pomocí GFP-nos3 3′UT mRNA a tetramethylrhodamine dextran pro značení PGC a somatických buněk. Vzniklo tak 12 chimér vyniklých transplantací blastomér, z nichž 8 se dožilo kulení. Ve vyvíjejících chimérách byly zřetelné dále se vyvíjející PGC a somatické buňky pocházející od donora. Transplantované PGC byly pozorovány během migrace až do usídlení v zárodečné rýze recipienta. Naše výsledky ukazují, že transplantace blastomér může být úspěšně aplikována u candáta obecného a mohou být tak užitečné pro produkci chimér zárodečné linie u okounovitých.

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LIST OF PUBLICATIONS

Peer-reviewed journals and their IF

Güralp H., Pocherniaieva K., Blecha M., Policar T., Pšenička M., Saito T. 2017. Migration of primordial germ cells during late embryogenesis of pikeperch *Sander lucioperca* relative to blastomere transplantation. Czech J. Anim. Sci. 62, 121–129. (IF 2016 = 0.8741)

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Books or chapters, monographs, textbooks

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International conferences

Güralp, H., Pšenička, M., Saito, T., 2016. A preliminary gene expression study on reference genes in pikeperch (*Sander lucioperca*). In: FABA 2016: International Symposium on Fisheries and Aquatic Sciences, Abstract Book, November 2–6, 2016, Antalya, Turkey, pp. 81–82. (Oral presentation)

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Güralp, H., Pocherniaieva, K, Blecha, M, Policar, T, Pšenička, M, Saito, T., 2013. Embryonic stages and Primordial germ gells development in pikeperch *Sander lucioperca* (Teleostei: Percidae). In: Diversification in Inland Finfish Aquaculture II (DIFA II). September 24–26, 2013, Vodnany, Czech Republic. (Oral presentation)

Güralp, H., Pocherniaieva, K., Blecha, M., Policar, T., Pšenička, M., Saito, T., 2013. Embryogenesis, visualization and migration of promordial germ cells in pikeperch *Sander lucioperca* (Teleostei: Percidae). In: 4th International Workshop on the Biology of Fish Gametes. September 17–20, 2013, Albufeira, Portugal. (Poster presentation)

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Hilal Güralp
Research department	2012–2017 – Laboratory of Germ cell RIFCH FFPW USB
Daily supervisor	
Supervisor	Assoc. Prof. Taiju Saito, Ph.D.
Period	1st October 2012 until 14th September 2017

Ph.D. courses	**Year**
Pond aquaculture	2013
Ichthyology and fish taxonomy	2013
Basic and scientific communication	2014
Applied hydrobiology	2014
Fish reproduction	2015
English language	2016

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2013 2014 2015 2016

International conferences	**Year**
Pšenička, M., **Güralp, H.**, Pocherniaieva, K., Linhartova, Z., Saito T., 2016. Generation of germline chimera in sturgeon. In: FABA 2016: International Symposium on Fisheries and Aquatic Sciences, Abstract Book, November 2–6, 2016, Antalya, Turkey, pp. 306-307. (Poster presentation)	2016
Güralp, H., Pšenička, M., Saito, T., 2016. A preliminary gene expression study on reference genes in pikeperch (*Sander lucioperca*). In: FABA 2016: International Symposium on Fisheries and Aquatic Sciences, Abstract Book, November 2–6, 2016, Antalya, Turkey, pp. 81–82. (Oral presentation)	2016
Güralp H., Pocherniaieva, K., Blecha, M., Policar, T., Pšenička, M., Saito, T., 2015. Embryogenesis and primordial germ cells development in pikeperch, *Sander lucioperca*. In: 5th International Workshop on the Biology of Fish Gametes. September 7–11, 2015, Ancona, Italy. (Oral presentation)	2015
Güralp, H., Pocherniaieva, K., Blecha, M., Policar, T., Pšenička, M., Saito, T., 2013. Embryonic stages and primordial germ gells development in pikeperch *Sander lucioperca* (Teleostei: Percidae). In: Diversification in Inland Finfish Aquaculture II (DIFA II). September 24–26, 2013, Vodnany, Czech Republic. (Oral presentation)	2013
Güralp, H., Pocherniaieva, K., Blecha, M., Policar, T., Pšenička, M., Saito, T., 2013. Embryogenesis, visualization and migration of promordial germ cells in pikeperch *Sander lucioperca* (Teleostei: Percidae). In: 4th International Workshop on the Biology of Fish Gametes. September 17–20, 2013, Albufeira, Portugal. (Poster presentation)	2013

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Warsaw University of Life Science, Faculty of Animal Science, Laboratory of Ichtybiology and Fishery, Warsaw, Poland (October 26 – December 4, 2015) Ph.D student / Studying development and differentiation process of gonads in sturgeon by histological and immunohistochemistrical methods.	2015
Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Science, Hokkaido University, Hakodate, Hokkaido, Japan (September 3–24, 2014) Ph.D student / Studying of in situ hybridization techniques.	2014

Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China (April 24 – May 29, 2014) Ph.D. student / Studying of transplantation of primordial germ cells technique, testicular stem cells culture and spermatogonia isolation in sturgeon and *in situ* hybridization techniques.	2014

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Hilal
Surname: Güralp
Title: Master of Science – M.Sc.
Born: 20th April, 1984, Istanbul
Nationality: Turkish

EDUCATION

2009–2012 M.Sc. – Istanbul University, Faculty of Fisheries, Aquaculture Division, Department of Fish Diseases

2003–2008 B.Sc. – Istanbul University, Institute of Science, Faculty of Fisheries, Aquaculture Division (scholarship)

PRESENT POSITION

2012 – present: Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW, www.frov.jcu.cz), Research Institute of Fish Culture and Hydrobiology (RIFCH), South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA), Laboratory of Germ Cells, Vodňany, Czech Republic.

Ph.D. COURSES Pond aquaculture, Fish reproduction, Ichthyology and fish taxonomy, Applied hydrobiology, Basic and scientific communication, English language.

SPECIALIZATION Transplantation of germ cells in fish

KNOWLEDGE OF LANGUAGES Turkish, English (IELTS certificate), Czech

RESEARCH STAY AND COLLABORATIONS

October 26 – December 4, 2015 Warsaw University of Life Science, Faculty of Animal Science, Laboratory of Ichthybiology and Fishery, Warsaw, Poland. Studying development and differentiation process of gonads in sturgeon by histological and immunohistochemistrical methods.

September 3–24, 2014 Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Science, Hokkaido University, Hakodate, Hokkaido, Japan. Studying of in situ hybridization techniques.

April 24 – May, 29, 2014 Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China. Studying of transplantation of primordial germ cells technique, testicular stem cells culture and spermatogonia isolation in sturgeon and *in situ* hybridization techniques.

CONSULTANCY OF STUDENT

Master student Ege Güngör, 2014–2015, Thesis title: Isolation of early stages of germ cells in pikeperch (*Sander lucioperca*).