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The foundation of maternal factors in sturgeon: from oocyte to embryo

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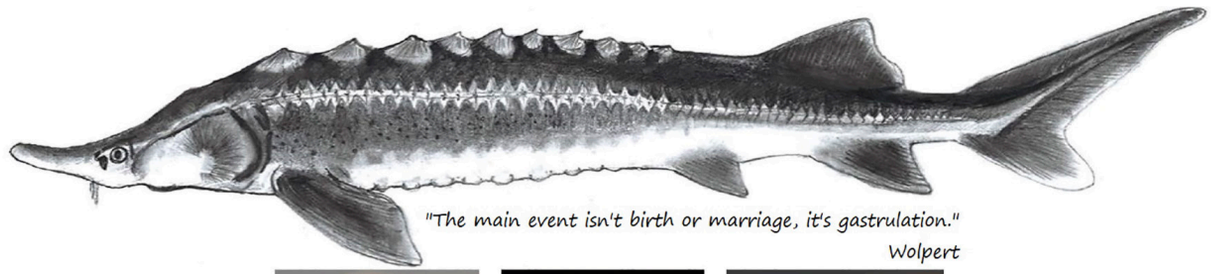
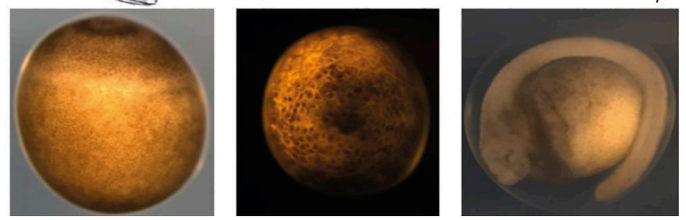
Základ mateřských faktorů u jesetera: od vajíčka po embryo



"The main event isn't birth or marriage, it's gastrulation."
Wolpert



Kseniia Pocherniaieva

Czech Republic, Vodňany, 2020

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Fertilization is a base of life. In animals, the fusion of male cell (spermatozoa) and female cell (oocyte) produce by this fusion a zygote cell. However, the contribution of gametes to the development of future organism is not equivalent, since the function of female gamete for later development is not only in the unification of the nuclei of both parents, but also in providing an ideal environment. The origin and the condition of the oocyte has a great influence on subsequent development of the embryo. To give the rise to a new organism and to stimulate the initial steps of embryonic development the egg must be maternally provisioned and contain all the necessary. It is already established that the early stages of embryonic development are entirely under the control of the maternal factor loaded to the oocyte during oogenesis. The proper function of maternal derivatives ensures the development, maturation, fertilization and early embryogenesis of oocytes. This maternal stock is represented by a high variety of messenger RNAs and proteins which exclusively stimulate the development of a transcriptionally inactive embryo (Andeol, 1994; Baroux et al., 2008).

Available tools for detection of intracellular mRNA localization include various molecular methods, like *in situ hybridization* or real-time polymerase chain reaction (PCR). To demonstrate the distribution of mRNAs in the sturgeon egg we applied novel technique of real-time PCR tomography (Sindelka et al., 2008). This comprehensive method has been selected as a reliable tool for the quantitative analysis of mRNA within a single cell. Moreover, this allowed us to study evolutionarily conserved similarities by comparing the distribution of maternal factors in sturgeon eggs with African clawed frog (*Xenopus laevis*) profiles (Sindelka et al., 2010; Sidova et al., 2015; Pocherniaieva et al., 2018).

Later, development control passes to the initially silent products of the zygotic genome and significantly affect maternal factors. The maternal to zygotic transition (MZT) is a separate developmental period that begins with the elimination of maternal transcripts, continues through the production of zygotic transcripts, and concludes with the first major morphological requirement for zygotic transcripts in embryo development (Baroux et al., 2008; Tadros and Lipshitz, 2009). The initiation of zygotic transcription during MZT is well-established and evolutionarily conserved in all species (Newport and Kirschner, 1982a; Kimelman et al*.,* 1987; Almouzni and Wolffe, 1995; Neusser et al*.,* 2007). Mid-blastula transition (MBT) in early embryogenesis has traditionally been defined as a time point characterized by cell cycle lengthening, loss of synchrony, acquisition of cell motility, and onset of zygotic gene transcription (Signoret and Lefresne, 1971; Gerhart, 1980; Newport and Kirschner, 1982a).

A study of these two processes of transition in sturgeon embryos during its early development, together with data on intracellular profiles in sturgeon eggs, provements about localization of primordial germ cells, and information on evolutionary similarities with different species, provide fundamental knowledges applicable for the conservation of endangered fish species *via* biotechnological approaches, such as surrogate production, somatic nuclear transfer, interspecific and intergeneric artificial hybrydization, host sterilization or genome editing.

Sturgeon

Sturgeon (order Acipenseriformes) is one the most ancient orders of basal actinopterygians, consisting of four main branches (Polypteriformes, Acipenseriformes, Lepisosteidae, and Amia) having evolved about 200 Mya (Bemis et al., 1997; Inoue et al., 2003). The Order Acipenseriformes is divided into two families, Acipenseridae (sturgeon) and Polyodontidae (paddlefish) (Berg 1940; Grande and Bemis 1991; Bemis et al., 1997). Taxonomically the Acipenseridae comprise two subfamilies, Acipenserinae including genera Acipenser and Huso which is phylogenetically primitive within Acipenseridae, and second subfamily is Scaphirhynchinae represented by the genera Scaphirhynchus and Pseudosaphirhynchus (Birstein, 1997).

The area of existence of this species is determined by their distribution in the Northern Hemisphere in the coastlines of North America, Europe and Asia, and also in the seas (Mediterranean, Caspian, Black and Azov), rivers and lakes (Dettlaff and Vassetzky, 1991; Bemis et al., 1997; Pikitch et al., 2005). Sturgeon is classified as both diadromous (migrations between marine and freshwater systems) and potamodromous (migrations within freshwater), but the ecological and biological plasticity of this species allows them to spend long period of their life in estuaries or completely assimilates in river deltas (Cherr and Clark, 1985; Dettlaff et al., 1993; Bemis and Kynard, 1997).

Nevertheless, dams constructed for hydroelectric generation affect water levels prevents migration of anadromous species to their feeding and breeding habitats (Billard and Lecointre, 2001). Pollution by industry and agriculture leads to disturbances in the development and functioning of the reproductive system and have posed a serious threat to Acipenseriformes (Ruban, 1997).

The sturgeon genome should probably be considered as a highly dynamic system, modeled by different evolutionary polyploidization events which accompanied a diversification within Acipenser (Vasiľev et al., 2009; Chen, 2010; Mayfield et al., 2011). Two different scales are currently being proposed for assessing the ploidy levels of Acipenseriformes: the 'evolutionary scale', which presumes tetra–ploid–octaploid–dodecaploid relationships among species, and the 'recent scale', which presumes diploid–tetraploid–hexaploid relationship. However, a review of all cytogenetic data summarizes that Acipenseriformes species can be divided into three groups: A with approximately 120 chromosomes and 3.2–4.6 picograms (pg) of DNA; group B with approx. 250–270 chromosomes and 6.1–9.6 pg of DNA; and group C consisting of a single species, the shortnose sturgeon, *Acipenser brevirostrum*, with 13.1 pg of DNA (Blacklidge and Bidwell, 1993) and a chromosome number of around 360 (Kim et al., 2005)

The unique easiness of hybridization of acipenserids was described by Russian ichthyologists Ovsyannikov and Zodraf more than 100 years ago (Birstein, 1997). Interspecific hybridization is a distinctive feature of Acipenseriformes compared to other vertebrates, in which hybridization between taxonomically distant species differing in the number of chromosomes is rare (Lagler et al., 1962; Arnold, 1997). Moreover, sturgeons demonstrate interspecific and intergeneric hybridization under artificial conditions (Arefjev, 1997; Flajšhans and Vajcová, 2000; Zhou et al., 2011, Havelka et al., 2013). Significant impact on this event is provided by special polyploidy state of different Acipenseriformes and high similarities in their genome (Birstein et al., 1997c).

The feature of the increased DNA content in the progeny of interspecific hybrids from different groups *A. gueldenstaedtii* x *A. ruthenus*, was used in Chapter 3 to study the timing and changes in *A. ruthenus* embryos and hybrid *A. gueldenstaedtii* x *A. ruthenus* embryos for the determination of MBT. (Pocherniaieva et al., 2019).

Violations during the zygotene stage of meiosis prophase I, due to the fact that the

chromosomes cannot pair properly, lead to the sterility of some hybrids (Pifferer et al., 2009). Therefore, for a long time it was assumed that hybrids between sturgeon with the same ploidy levels are capable of producing progeny, and those resulting from hybridization of parental species of different ploidy levels are infertile (Williot et al., 1993; Billard and Lecointre, 2001). Nevertheless, our colleagues have already demonstrated that progeny of spontaneous triploid male *A. baerii* is fully fertile. Fertilization of ova of *A. baerii* and *A. gueldenstaedtii* with normal ploidy with sperm of the spontaneous triploid *A. baerii* produced fully viable progeny with ploidy level intermediate to those of the parents (Havelka et al., 2013).

Most sturgeon species are currently considered to be threatened with extinction and according to IUCN, sturgeon as live fossil are "more critically endangered than any other group of species" (IUCN Red List, 2012). Reduction of populations of wild sturgeon in recent decades is associated with a sharp increase in caviar trade in all around the world (Ludwig, 2008). Chondrostei roe sold as black caviar is the most luxurious animal product in international trade (Birstein, 1997b; Ludwig, 2008). Concerns of overharvesting and the conservation status of many of the 27 extant species of Acipenseriformes led to all species being included on the CITES Appendices in 1998 (Ludwig, 2008). Appendix I (two species) or II (25 species), only two species are considered to be in Lower Risk by IUCN, four of the nine US taxa and one Caspian species are protected under the Endangered Species Act, and local extinctions recorded for 19 of 27 species (Pikitch et. al., 2005). Despite the introduction of a trade control and regulation system, illegal fishing and poaching continue to pose a threat to many sturgeon populations (Ludwig, 2008; De Meulenaer and Raymakers, 1996). The experience of North America shows that captive breeding and sustainable fish production are being introduced as an alternative tool for the restoration of endangered fish species, with great attention being paid to the release of programs, improving domestic, international fisheries management and the significant habitat of fish populations (Pikitch et. al, 2005; Webb and Doroshov, 2011).

The fact that most sturgeon species are currently considered to be threatened with extinction together with their commercial and ecological value has led to growing interest in the study of possibilities of the restoration of fish stocks using novel bio-engineering approaches applied in other fish species (Yamaha et al., 2007; Yoshizaki et al., 2002; Yoshizaki, 2005). An example is the induction of chimerism by transplantation of germ stem cells in critically endangered sturgeons. The transfer of primordial germ cells between different sturgeon species might reduce the time required for production of donor gametes by surrogate parents (Psenicka et al., 2015). Complete host sterilization is essential for sturgeon surrogate production, so our colleagues have developed several methods for removing germ cells from the host: morpholino knockdown, use of ultraviolet radiation and the latest CRISPR / Cas9 genome editing technology (Linhartova et al., 2015; Saito et al., 2018; Baloch et al., 2019).

Another promising technique potentionaly applicable in fish restoration is realized through the nuclear transfer from somatic cells to oocytes. Nuclear transfer carried out on the goldfish oocytes (*Carassius auratus*) reveals the possibility of utilization of cryopreserved somatic cells for fish reconstruction (Le Bail, 2010). The multiply somatic cell nuclear transfer (mSCNT) have been also applied for sturgeon species - Russian sturgeon (*Acipenser guldenstaedti*) and the beluga (*Huso huso*). The mSCNT embryos displayed normal development and most of them even went through critical stage of MBT. A species-specific analysis showed bearing both the recipient and donor genome of mSCNT embryos. The results of this research demonstrate the success of the nuclear transfer and its potential for the conservation of sturgeon species (Fatira et al., 2019).

Basics of oogenesis and maturation in sturgeon

The ability of egg to be fertilized and subsequently develop into competent organism is determined by the successful passage of the steps of oogenesis. Generally, to provide energy and metabolism of the oocyte and the developing embryo, it is necessary to create extensive reserves of cytoplasmic organelles, nutritive compounds, enzymes for the synthesis of proteins, nucleic acids, carbohydrate and fat metabolism. In addition, it is essential to ensure the appropriate level of synthesized mRNA and proteins that define both the transformation of the oocyte into the egg and the initial stages of embryonic development in the process of oogenesis.

The extensive data on the structure and characteristics of sturgeon oocyte at all stages of development are presented in the studies of Dettlaff et al. (1981). The maturation stage can be defined as a hormone-dependent period without any drastically changes in the structure of oocyte.

In all metazoans, feature that also determines the progress of oocyte maturation is the positioning of the nucleus into the cortical position. The position of the germinal vesicle (GV), the rounded or oval nucleus in the oocytes of Acipenseridae is determined by the location near the surface in the central part of animal pole (Dettlaff et al., 1981). The identification of oocyte polarization index (PI) plays a decisive role in determining of the potentially spawnable females. Currently, one of the most reliable method for identification of PI was introduced by Rodina and allows to process a large number of oocytes with high accuracy (Rodina, 2006).

Maturation of the oocyte is achieved through the passage of the growth phase under the influence of external triggers and intracellular events. The last one realized through the activation of maturation promoting factor which is a key regulator of both meiotic and mitotic cell cycles (Wu et al., 1997). In most animals the sperm penetrates the egg after a certain period of time following the meiotic arrest.

The subsequent changes during maturation are related to the modification of the oocyte surface layer by including of cortical granules (Skoblina, 1968, 1969; Smith et al., 1968; Smith and Ecker, 1969a, b; 1970; Murata, 2003). Thus, the membrane structure becomes denser. These changes have a great impact on the process of fertilization in sturgeons, since the launching of a cortical reaction is directly associated with the prevention of polyspermy in fish (Ginzburg, 1972; Psenicka et al., 2010). Capacity for cortical response in the oocytes of the sturgeon appears during metaphase I (Dettlaff et al., 1981). Moreover, composition of the cytoplasm itself undergoes changes related to hydration, which in turn affects the sensitivity of the oocyte to temperature factors (Dettlaff et al., 1980; Skoblina, 1969).

Ovulation and maturation of oocytes are two relatively distinct processes, but their course can be disturbed by the influence of many negative factors, both physical and chemical (Wright, 1945; Dettlaff et al., 1980; Reynhout et al., 1975). Unfortunately, such violations lead to ovulation of immature oocytes or even parthenogenetic effect during cleavage (Dettlaff et al., 1981).

Oocyte polarity (establishment of mRNAs and proteins, localized cytoplasmic determinants)

The phenomenon of RNA localization is evolutionarily ancient strategy and highly conserved feature among many animals. One of the most striking characteristics is the position of the nucleus in the center of the oocyte, but as oogenesis progresess, the oocyte nucleus moves to the cortex, and the side where the nucleus is located is usually defined as the animal pole. The opposite side of the egg is called the vegetative pole and is predominantly filled

with yolk. It should be noted that in addition to nutritional function, this region is the site of primary localization of primordial germ cells (PGCs), and then this pole differentiates into the embryonic body (Saito et al., 2014). The color of the different poles of the egg is not the same and reflects the polarity of its internal structure.

Generally, the animal region is characterized by a distinct pattern with a light polar spot in the center surrounded by a ringlike accumulation of pigment granules (Fig. 1). Vegetal pole is darker and visually separeted from the animal region by another pigment circle (Dettlaff et al., 1981) The color patterning described above is generalized for all Acipenseriformes and can vary not only between different species, but even among different females.

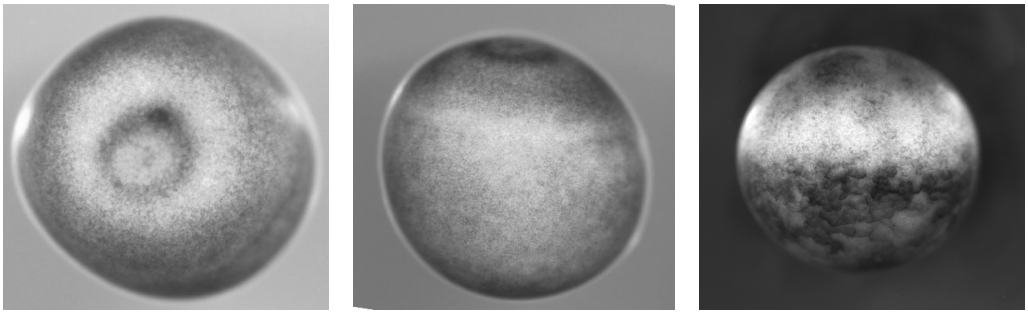

Figure 1. *Developmental pattern of a normally dividing A. ruthenus embryo: 1-cell upper view, 1-cell side view and 1000-cell stage (Pocherniaieva, K. not published).*

The animal–vegetal axis is patterned exclusively by maternal factors, the later developing dorsal–ventral axis depends on formation of the animal–vegetal axis and requires both maternal and zygotic contributions in teleosts and amphibians. The most evident effect of axis patterning in body plan of embryo is formation of the first body axis determined based on animal-vegetal pole orientation. According to Huynh and St. Johnston, orientation of the last mitosis and the position of the centrosome are evolutionarily conserved during oogenesis to set up animal-vegetal polarity (Huynh and St. Johnston, 2004).

During the prolonged prophase I arrest in meiosis, oocytes produce or accumulate mRNAs and proteins necessary to complete later aspects of oogenesis and early development of the embryo. It has been demonstrated (Wasserman et al., 1972; Roeder, 1974; Hollinger and Smith, 1976) that already toward the period of maturation the germinal vesicle (GV) of clawed frog oocytes contain a large amount of RNA-polymerases. The swingeing majority of this RNA-polymerases is mounted in the nucleus, and only around 10% is distributed in the cytoplasm. After the decay of the GV envelope and the transfer of nucleoplasm to the cytoplasm, the RNA polymerase is retained and in inactive state and during the early embryogenesis, it appears to migrate to the nuclei (Hollinger and Smith, 1976). Thus, RNA-polymerases appear in the oocyte during the period of growth and during the period of maturation are preserved only to the beginning of embryogenesis.

The pre-pattern of differentially localized mRNA and resultant protein deposition is the foundation of the precisely controlled process of cell differentiation and germ layer patterning during embryo development. A slight alteration in the balance of maternal factors can leads to changes in cell differentiation (Gurdon, 2006). Maternal mRNAs are the most suitable candidates for determinants, since they can be translated into many copies of regulatory proteins (Jeffery, 1984). The proper localization ensures the function of certain substances in the subcellular domains.

The first report dedicated to embryonic development and pre-larvae stage of sterlet *Acipenser ruthenus* were done by Kowalewsky in 1870, and now among all Acipenseriformes sterlet can be proposed as an ideal model for research (Dettlaff et al., 1993; Saito et al., 2014; Psenicka et al., 2015; Linhartova et al., 2015; Saito et al., 2018; Chen et al., 2018; Fatira et al., 2019; Baloch et al., 2019).

In the second half of the twentieth century, the first descriptions of sturgeon embryogenesis appeared on the example of *Acipenser stellatus* and later were complemented by descriptions of other Acipenseroformes. However, embryonic development and structure of embryo itself is very similar among genera *Acipenser* (Ginsburg and Dettlaff, 1991; Park et al., 2013; Bolker, 1994).

It is interesting to note that embryogenesis of sturgeon resembles characters of embryogenesis of amphibians in many aspects (Bolker, 1993; Bartsch et al., 1997). In sturgeon and amphibians eggs, the yolk is organized in the form of platelets with a crystalline core, opposite to the teleost in which yolk is organized in the form of lipoprotein spheres (Kilarski and Grodzinski, 1969).

Holoblastic cleavage in early development of sturgeon (Macgregor, 1972) and amphibians (Elinson, 2009) is similar due to the presence in egg cytoplasm of yolk granules, while most teleosts exhibit meroblastic cleavage (Wourms, 1997). Thus, the cleavage of blastomeres in the poles of the sturgeon embryo will be significantly different due to the uneven distribution of lipid and deutoplastic inclusions in the structure of the oocyte (Colombo et al., 2007). The blastomeres of animal pole characterized by a convex shape and minor discrepancies in size. The number of cleaved blastomeres in vegetal hemisphere reduced as it starts after the first division, which is offset by the size of these cells (Ginsburg and Dettlaff, 1991; Park et al., 2013). As mentioned in the previous paragraph, PGCs originate from the vegetative pole (VP) of sturgeon eggs and then migrate along the yolk cell mass in the direction of the gonadal ridge, which resembles the migratory pattern of amphibians (Fig. 2). In the teleosts, for example in zebrafish, PGCs are located in the marginal region of the blastodisc, in close proximity of the syncytial layer of the yolk, which plays an exclusively nutritional role and subsequently does not differentiate into the embryonic body.

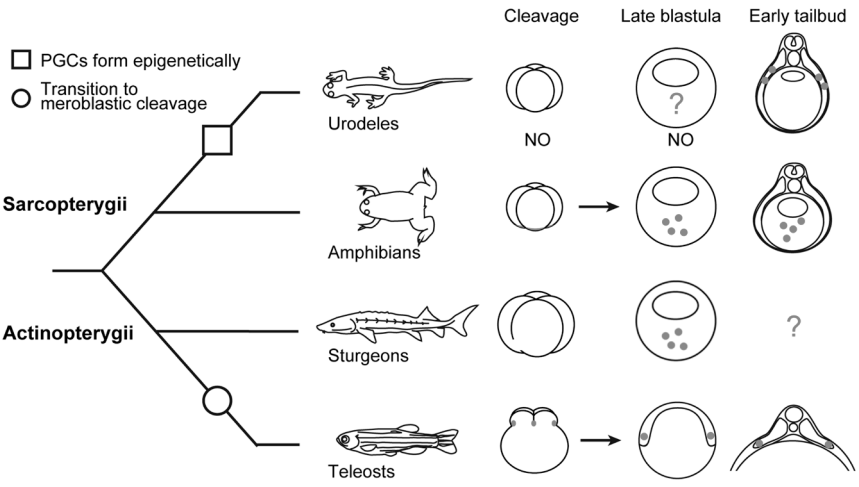

Figure 2. *Schematic representation of embryo cleavage patterns and formation of primordial germ cells (PGCs) in selected vertebrates (figure is modified from Saito et al., 2014).*

The initial step in embryogenesis is the activation of egg upon fertilization, followed by the rapid division of the blastomeres. The position of cleavage furrows strictly follows the Hertwig rule (long axis rule), according to which the spindle axis is oriented in the direction of the maximum length of the cytoplasm free of inclusions (Dettlaff et al., 1981). Irregularities in the nature of cleavage patterns in the eggs of sturgeon may be caused by two different processes: polyspermy or parthenogenesis.

The presence of superfluous blastomeres is an atypical pattern of polyspermic embryos. Fertilization by more than one spermatozoa which can penetrate through the special micropilylar channels in the oocyte membrane of sturgeon and thereby cause polyspermy (Fig. 3) (Ginsburg, 1972; Cherr and Clark, 1982; Iegorova et al., 2018). Several organisms such as insects, urodele amphibians, reptiles, and birds exhibit physiological polyspermy (Elinson and Pasceri, 1989; Iwao, 2000a; Wong and Wessel 2006; Snook et al., 2011). The number of micropiles in sturgeon egg varies from 1–2 to 43 in different females (Poduska, 1992). As discussed above, the blocking of polyspermy in fish is implemented by depolarization of the egg's plasma membrane by means of cortical granules (Dale et al., 2014; Jaffe, 1976). Psenicka et al. (2010) reported that a cytoplasmic projection in the sturgeon egg created after fusion with the spermatozoon rapidly expands concentrically from the point of first spermatozoon penetration toward the vegetal pole, blocking entry by additional spermatozoa into other micropyles. In zebrafish the fails on the establishment of animal-vegetal (AV) polarity both within the maturing oocyte and in the surrounding follicle cells by the asymmetric redistribution of cellular components and molecules might lead to the multiply micropile formation, which subsequently induces polyspermy (Lindeman and Pelegri, 2010). The ability of eggs to be activated without fertilization is a characteristic of Acipenseridae. Parthenogenetic egg (Fig. 3) activation in sturgeon embryos leads to irregular cleavage with arrest in development, but the mechanism of this process remains unclear (Dettlaff, 1993).

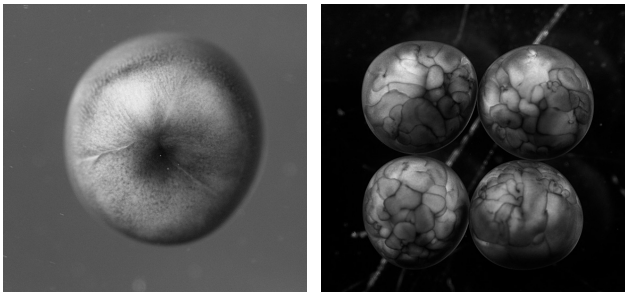

Figure 3. *Physiological irregularities in developmemt of A. ruthenus embryous: 3-cell polyspemy embryo, parthenogenetic embryous (Pocherniaieva, K. not published).*

During blastulation, a new blastomeres are formed, these are hardly countable in late blastula stage and indistinguishable from one to another in animal pole due to its small size. Large blastomeres of the vegetal hemisphere are located on top, while the small blastomeres are located around the marginal zone (Park et al., 2013). Fate mapping reveals that all blastomeres contribute to the germ layer formation of the cell lineage patterning in vertebrates. In addition, vegetal blastomeres of holoblastic embryos have been generally assumed to contribute also to embryonic endoderm, but in teleosts, the vegetal yolk cells never differentiate to any embryonic layers and play only a nutritional role (Bolker, 1993a, b; Pelegri, 2006).

Germplasm and primordial germ cells

Fish, like many other animals, have two major cell lineages, namely the germline and somatic cells. The processes underlying the specification of these two cell lines excite the minds of many scientists. As discussed earlier in the chapter above, the sturgeon oocyte contains impressive number of yolk cells forming vegetative extraembryonic tissues that set apart the two main lineages in terms of the body of the Acipenseridae oocytes (Kilarski and Grodzinski, 1969; Saito et al., 2014). Here I will focus on germ cell lineage under authority of response for unique proliferative and differentiated capacities. Primordial germ cells are the only embryonic cells having a potential to transmit genetic information to the next generation (Weismann, 1893). PGCs give rise to male and female gametes. In all systems, precursors of germ-line form far from the site of the developing gonads and migrate to the sites of developing ovaries or testes.

The first attempt to produce a germline chimera using surrogate parents in Siberian sturgeon was performed by Psenicka et al. (2015). Successful isolation and transplantation of the early stage of oogony and spermatogonia, as well as their proliferation in the recipient, can be considered the solid basis for the development of gene banking and cryopreservation or, in particular, for the production of donor gametes through germ-line chimeras. Prior to utilization of surrogate production in rare or endangered fish species as a sturgeon, it is essential to investigate fundamental insights of sub-cellular expression profiling of developmental mRNAs, since it subsequently affects specification and migration of the germ cells.

Previous research of PGCs culture provided histological structure description. In this case, we clearly have known that the cells of this type have comparatively large (10-20 μm) round shape with large nucleus (6–10 μm) which contains one or two prominent nucleoli within the evenly distributed chromatin. Also PGCs have relatively little cytoplasm (i.e. a high nuclear-cytoplasmic ratio) and lucid nuclear membrane. Using these criteria, PGCs could be traced back to the late blastula or somitogenesis stages (Yamaha et al., 2010). In the framework of studies on the development of PGCs, identification of their migration and distribution structure in embryos, several methods of visualization of these cells have been established in sturgeon species. The cell labeling substance-based (fluorescein isothiocyanate [FITC]-dextran) and RNA-based (GFP-*nos 1* 3′-UTR mRNA) PGC-labeled method remains one of the most reliable, since the sexual maturation of sturgeon complicates and practically makes it impossible to generate solid transgenic strains in sturgeons (Psenicka et al., 2015).

Studies on migratory activity of PGCs in sturgeons revealed the pathway which is remarkably similar to the teleost, however the place of their specification resemble that in *Xenopus laevis* (King et al., 2005; Kloc et al., 2004; Lindeman and Pelegri, 2010; Saito et al., 2014). The localization of maternally derived determinants, so-called *preformation*, is one of two modes of PGC specification common to a range of model organisms including nematode *Caenorhabditis elegans,* fruit fly *Drosophila melanogaster,* frog *Xenopus laevis,* and chicken *Gallus gallus*. In mammals, PGC specification occurs *via* epigenesis through signals from neighboring somatic cells (Nieuwkoop and Sutasurya, 1981; Extavour and Akam, 2003). The specification mode of primordial germ cells provides robust evidence of similarities between amphibians and actinopterygii. Recently, Saito et al. (2014) showed that sturgeon primordial germ cells (PGC) are generated at the vegetal hemisphere by germplasm, as is seen in *Xenopus laevis*. During zebrafish embryogenesis, germplasm localizes to the first two cleavage furrows, which is essential for the specification of four clusters of primordial germ cells (Raz, 2002). The germplasm is specific area of the cytoplasm that determines the fate of the germ cell. The autonomy of PGCs from neighboring cells is provided by suppressive transcription factors contained in the germplasm (Venkatarama, 2006). It is assumed that such an important

function of germplasm conserved across several species during embryogenesis (Strome and Lehmann, 2007).

Recent data on the structure of the germplasm clearly determined its structure in sturgeon, which is characterized as nuage, also called a mitochondrial cloud (Saito et al., 2014). This is a membrane-less cytoplasmic organelle containing RNAs and proteins (Hamaguchi, 1982). It has been shown that germ line-specific electron-dense structures represent a storage of RNAs and proteins which may be essential for differentiation and/or determination of PGCs perhaps by regulating germ line specific translation and/or transcription. Despite this, it still remains unclear what is the exact profile of distributed RNAs and proteins in sturgeon oocytes, as well as the quantification of markers responsible for the induction of proliferation and subsequent determination of PGCs was not determined. On the other hand, the mechanism which governs the specification of PGCs upon activation of zygotic genes, as well as, regulation of their migratory activity during MBT is still unknown.

Mid-blastula transition/ Maternal–Zygotic Transition

Rapid cell division ends shortly after a breach of the nucleus to cytoplasm ratio, which leads to the rearrangement of the cell cycle. During this time divisions of cells without any progress in size of embryo regulated by cytoplasmic determinants. Mid-blastula transition (MBT) in early embryogenesis has traditionally been defined as a time point characterized by cell cycle lengthening, loss of synchrony, acquisition of cell motility, and onset of zygotic gene transcription. Experiments with mechanical compression of the cytoplasm showed that a reduction of certain compounds subsequently affected the time of transition (Dettlaff, 1964; Newport and Kirschner 1982a, b; Edgar et al., 1986).

Early studies in fish and amphibians referred the switch from the cleavage type to mitosis with inclusion of gap phases involves zygotic genome activation. Among the varied approaches to identify the transition, the experiments with mechanical constriction of the cytoplasm, induction of polyspermy, and injections of exogenous nonspecific DNA were performed (Newport and Kirschner, 1982a; Etkin, 1988; Kane and Kimmel, 1993; Lee et al., 2014).More recent researches treat maternal to zygotic transition (MZT) as a separate developmental period that begins with the elimination of maternal transcripts, continues through the production of zygotic transcripts, and concludes with the first major morphological requirement for zygotic transcripts in embryo development (Tadros and Lipshitz, 2009).

The maternal to zygotic transition is not a single event but realized through the set of several steps. This transition begins with activation of the egg and extends through activation of zygotic gene expression and culminates with the complete elimination of maternal products. The extent of the turnover of maternal products and the developmental stage when maternal clearance initiates and concludes varies (Baugh et al., 2003; Hamatani et al., 2004; De Renzis et al., 2007). In some animals, maternal products are not completely eliminated until stages well after zygotic gene expression begins.

Maternal to zygotic reprogramming is characterized by degradation of maternal factors and activation of zygotic transcripts. The onset of zygotic genome activation (ZGA) is governed by an integrated model on several levels: histone modifications directly define chromatin status for transcription, increase in nucleus to cytoplasm ratio, a developmental timer regulating degradation of Cyclin A and E1 proteins, and lengthening of the cell cycle (Howe et al*.,* 1995; Howe and Newport, 1996; Stack and Newport, 1997; Zegerman and Diffley, 2007). Although the initiation of zygotic gene transcription is evolutionarily conserved, its mechanism is species-specific.

Epigenetic Modifications and Zygotic Genome Activation

Activation of major zygotic transcript onset after several cleavage stages and in some organisms even coincides with the desynchronization of cell cycle and acquisition of cell motility. In early development of mammalian and nematodes this transition achieved in relatively short time which is varying from the one cell stage to the four cell stage. The concept underlying the machinery of zygotic genome activation related to extensive network of the transcription suppression factors. This specific repressors and epigenetic modifications constitute the integrated model which is ensure transcriptional embryonic genome silencing on the early stages of development. Almouzni and Wolffe (1995) hypothetised that the titration of inhibitors by DNA and deficiency in the activity of transcriptional activators prior to the MBT are the main features of temporal regulation of transcription during early embryogenesis in *Xenopus*.

The DNA demethylation kinetics and modification of chromatin are major mechanisms in the epigenetic reprogramming and play the crucial role during embryogenesis due to global pluripotency state establishment in the newly formed organism (Prioleau et al., 1994). The reprogramming in primordial germ cells occurred *via* realization of the proper demethylation as well. Another factor affect to onset of pluripotency through the blocking of gene expression is histone modification. In *Xenopus,* repression of TATA-binding protein (TBP) activity conditioned by large excess of maternal histones stored in the egg (Adamson and Woodland, 1974; Laskey et al., 1977; Woodland and Adamson, 1977; Veenstra et al., 1999), while in zebrafish embryo translation of that protein is only required for degradation of some maternal mRNAs (Ferg et al., 2007). TATA-binding protein is required for establishment of zygotic gene expression and regulation and also participates in transcription initiated by RNA polymerases I, II, and III, which translated from maternal stock prior to mid-blastula transition (Hernandez, 1993; Veenstra et al., 1999; Müller et al., 2001; Martianov et al., 2002).

Maternal Control after Zygotic Genome Activation

As mentioned above, the formation of embryonic axes solely depends on the localization of maternal factors and subsequently influences the zygotic genome activation. But the influence of maternal genes is exerted even after the launching of its own zygotic transcription. The process of degradation of maternal factors has been little studied in prehistoric species of fish, therefore the mechanics of protection of maternal products also remain unclear. We can anticipate that *de novo* zygote products are puffed up in close conjunction with the mother products in order to promote normal development. In some cases, the continuing maternal function is completely compensated, and in other cases it delays embryonic arrest until later developmental stages, when the maternal product is ultimately depleted. However, the first step in clarifying the ideas of transition during early development is entirely based on the exact spatial distribution of maternal factors.

Objectives of the Thesis

In present study, we aimed to exhibit distribution maternal factors of sturgeon egg responsible for the formation and migration of PGCs, together with identification of transition points, since it can help to understand gametogenesis, sex differentiation, PGCs evolution and basics of normal development in sturgeon.

The aims of the present study:

1. To reveal fundamental architecture of maternal messenger RNAs in sturgeon oocyte via method of quantitative polymerase chain reaction tomography,
2. To clarify the origin of germplasm in body plan of sturgeon oocyte and evaluate the impact of germplasm localization on germ cells fate,
3. To characterize the transition from maternal control to activation of zygotic genome in sturgeon embryos,
4. To identify the point of mid-blastula transition in sterlet *Acipenser ruthenus* and *A. ruthenus* x *Acipenser gueldenstaedtii* hybrid,
5. To compare mRNA profiles in sturgeon with available patterns in the frog oocyte.

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CHAPTER 2

COMPARISON OF OOCYTE mRNA LOCALIZATION PATTERNS IN STERLET *ACIPENSER RUTHENUS* AND AFRICAN CLAWED FROG *XENOPUS LAEVIS*

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Comparison of oocyte mRNA localization patterns in sterlet *Acipenser ruthenus* and African clawed frog *Xenopus laevis*

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Abstract

In oocytes, RNA localization has critical implications, as assembly of proteins in particular subcellular domains is crucial to embryo development. The distribution of mRNA molecules can identify and characterize localized transcripts. The goal of this study was to clarify the origin of primordial germ cells in the oocyte body plan and to reveal the generation of cell lineages by localized RNAs. The distribution of 12 selected mRNAs in sterlet *Acipenser ruthenus* oocytes was investigated by qPCR tomography and compared with known patterns of mRNA localization in *Xenopus laevis*. We investigated the distribution of two gene clusters in the ooplasm along the animal–vegetal axis of the sturgeon oocyte, both of which showed clearly defined intracellular gradient pattern remarkably similar to their distribution in the frog oocyte. We elucidated the localization of sturgeon egg germplasm markers belonging to the vegetal group of mRNAs. The mRNAs coding *otx1*, *wnt11*, and *veg1* found to be localized in the sturgeon animal hemisphere are, in contrast, distributed in the vegetal hemisphere in amphibian. Actinopterygii and Sarcopterygii, two major lineages of osteichthyan vertebrates, split about 476 Ma (Blair & Hedges, 2005), albeit basal lineages share conserved biological features. Acipenseriformes is one the most basal living lineages of Actinopterygii, having evolved about 200 Ma (Bemis, Birstein, & Waldman, 1997), contemporaneous with modern amphibians (Roelants et al., 2007).

KEYWORDS

acipenser ruthenus, oocyte, primordial Germ Cells, qPCR, RNA localization, sturgeon, xenopus laevis

1 | INTRODUCTION

A decrease in wild sturgeon populations in recent decades has led to growing interest in the use of germ cells in reproduction to restore fish stocks (Okutsu, Suzuki, Takeuchi, Takeuchi, & Yoshizaki, 2006, Robles et al., 2016, Yamaha et al., 2010). The effective application of such embryo engineering to sturgeon requires detailed knowledge of its embryonic development.

Studies of sturgeon embryogenesis will also provide valuable comparisons with ontogeny of actinopterygians and amphibians (Saito et al., 2014). The embryogenesis of sturgeon resembles that of amphibians in many aspects. Holoblastic cleavage in early development of sturgeon (Macgregor, 1972) and amphibians (Elinson, 2009) is similar, while most teleosts exhibit meroblastic cleavage (Wourms, 1997). The yolk volume in sturgeon eggs is different from that in *Xenopus*. Sturgeon eggs are relatively large, due to the yolky cell mass at the vegetal hemisphere and vegetal pole blastomeres that are nutritive and do not differentiate into embryonic tissue (Saito et al., 2014), while mesodermal and endodermal tissues of *Xenopus* embryos develop from the vegetal hemisphere (Clements, Friday, & Woodland, 1999). There are several types of holoblastic cleavage patterns depending on the yolk size. Different embryos of isolecithal type of holoblastic cleavage have little yolk compared to sturgeon embryo, which can be explained by rapid embryonic development or by external nourishment. The cleavage asymmetry of these embryos is characterized by four variable patterns: radial (sea urchin, sea star), bilateral (tunicates), spiral (molluscs, ringed worms), and rotational (mammals, nematodes). The lower fishes and amphibians share radial symmetry with unequal blastomere division.

Acipenseridae resemble Amphibia in many cytological aspects, including oogenesis. Oocytes of acipenserids are more similar to amphibian oocytes than to those of teleosts (Raikova, 1976). Oocytes

of acipenserids and amphibians exhibit similar structure of yolk plates and cortical granules, absence of ribosomes in previtellogenesis, extrusions of nucleolar material into the cytoplasm, and dense material cementing mitochondria. The oogenic nucleoli in acipenserids also closely resemble those of amphibians (Macgregor, 1972). The structure of Balbiani cytoplasm of previtellogenic oocytes in Russian sturgeon is homologous to that in *Xenopus* (Zelazowska, Kilarski, Bilinski, Podder, & Kloc, 2007). Properly functioning Balbiani bodies support organization and transport of RNA and organelles within the oocyte (Kloc, Bilinski, & Etkin, 2004, Wilk, Bilinski, Dougherty, & Kloc, 2005), which subsequently encode germ cells.

Localization of RNA in oocytes is critical to early embryogenesis, including germline specification by the germplasm (Cuykendall & Houston, 2010, King & Zhou, 2004, Machado et al., 2005). The specification mode of primordial germ cells provides robust evidence of similarities between amphibians and actinopterygii. Recently, Saito et al. (2014) showed that sturgeon primordial germ cells (PGC) are generated at the vegetal hemisphere by germplasm, as is seen in *Xenopus laevis*. The localization of maternally derived determinants, so-called *preformation*, is one of two modes of PGC specification common to a range of organisms including nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster*, frog *Xenopus laevis*, and chicken *Gallus gallus*. In mammals, PGC specification occurs via epigenesis through signals from neighboring somatic cells (Extavour & Akam, 2003, Nieuwkoop & Sutasurya, 1981). The localization of PGCs in sturgeon, as well as the manner of specification, is identical to that in anurans (Saito et al., 2006, Saito et al., 2014, Whitington & Dixon, 1975); whereas the PGC migration pathway in the sturgeon embryo is hypothesized to be identical to that seen in many teleosts. It has been suggested that sturgeon PGCs develop from the vegetal yolk cells, that is, in the extra embryonic region.

RNA localization is a common mechanism for establishing cell determination and function (King, Messitt, & Mowry, 2005, Singer, 1992). In oocytes, large deposits of maternally synthesized mRNAs show animal, vegetal, and extreme vegetal gradients. Some RNAs are transported to, and accumulate at, the vegetal pole, as in the METRO pathway in *Xenopus*, while others, to ensure future embryo development, are localized at the animal pole (Kloc, Larabell, Chan, & Etkin, 1998, Sidova, Sindelka, Castoldi, Benes, & Kubista, 2015). The pre-pattern of differentially localized mRNA and resultant protein deposition is the foundation of the precisely controlled process of cell differentiation and germ layer patterning during embryo development.

Due to the uneven distribution of lipid and deutoplastic inclusions in the structure of the oocyte blastomeres of animal pole are characterized by a convex shape and minor discrepancies in size (Colombo, Garvey, & Wills, 2007, Dettlaff, Ginsburg, & Schmalhausen, 1993, Dettlaff & Vassetzky, 1991, Park, Lee, Kim, & Nam, 2013). This large amount of yolk in the vegetal portion causes the cleavage to proceed at a slower rate (Colombo et al., 2007). During blastulation are uniformed a new blastomeres, which are hardly countable in late blastula stage and indistinguishable from one to another in animal pole due to small size. Large blastomeres of the vegetal hemisphere are located in the near the top itself, while the small blastomeres are located around the marginal zone (Park et al., 2013).

Information on sturgeon embryonic development and its molecular background can be gained through screening intracellular geometry and mechanisms of fundamental processes, such as RNA organization. Comparative analysis of intracellular mechanisms of morphogenesis in the distantly related species *Xenopus laevis* and *Acipenser ruthenus* can reveal significant aspects of early development that have been conserved during evolution.

The goal of this study was to clarify distribution of selected mRNAs in sterlet *Acipenser ruthenus* oocytes, especially germ cell related genes, to investigate where PGCs are localized in the body plan of the sturgeon oocyte, the function of the yolky vegetal region of the sturgeon embryo, and the difference in the fate map of sturgeon and *Xenopus* genes.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Experimental procedures were carried out in accordance with the Czech Law 246/1992 on animal welfare, for which the authors possess a certificate according to section 17 of the law. Protocols underwent review by the Animal Research Committee of the Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Authorization for use of experimental animals No. 53100/2013-MZE-17214 valid from August 30, 2013 to August 30,2016.

2.2 | Oocyte collection, sectioning, and RNA extraction

Ovulation was induced in sterlet *A. ruthenus* with intramuscular injections of carp pituitary extract powder dissolved in 0.9% (w/v) NaCl solution at an initial dose of 0.5 mg/kg body weight, followed by a second injection at 4.5 mg/kg body weight 12 hr after the first injection. The ovulated eggs were collected from three females via a minimally invasive incision of the oviduct 18–20 hr after the second injection.

Four oocytes were optimally oriented, and each was separately embedded in a drop of Sakura Tissue-Tek optimal cutting temperature compound (4583) on a precooled dissection block. The block was placed for 10 min in a cryostat chamber (−17 to −18°C) to equilibrate the temperature of samples. The temperature of the sectioning knife was −18°C. Oocytes were cut into eighty-five 30 μm sections, across the animal–vegetal axis. Slices were divided into five tubes with 16–17 consecutive slices in each group, corresponding to five sequential segments of oocyte: 1, animal segment; 2, animal-marginal segment; 3, marginal segment; 4, marginal-vegetal segment; and 5, vegetal segment. TRIzol reagent (Invitrogen) (400 μl) was added to each tube to extract total RNA. Samples were homogenized by vortexing for 2 min and incubated for 5 min at room temperature to dissolve

completely. Manufacturer's instructions were followed during the isolation, with the addition of 10 μg of glycogen (Sigma–Aldrich) to the isopropanol to enhance RNA yield. Precipitated RNA was re-dissolved in 15 μl of RNase-free water, and the concentration was determined with the Nanodrop® ND1000 quantification system (Nanodrop Inc.).

2.3 | Reverse transcriptase-polymerase chain reaction

The SuperScript™ III Reverse Transcriptase kit (Invitrogen) was used for cDNA synthesis. Total RNA (50 ng), 0.5 μl of a 1:1 mixture of oligo-dT and random hexamers (50 μM each), 0.5 μl of dNTPs (10 mM each), and 0.5 μl of RNA spike (in vitro-transcribed artificial RNA with 3′ poly(A) tail and 5′ cap, TATAA Biocenter) were mixed with sterile water to a total volume of 6.5 μl. Samples were incubated for 5 min at 75°C, followed by 20 sec at 25°C, and cooled at 4°C for 1.5 min. The reaction mix (10 μl) contained 100 U SuperScript III Reverse Transcriptase, 20 U recombinant ribonuclease inhibitor (RNaseOUT, Invitrogen), 0.5 μl of 0.1 M DTT, and 2 μl of 5× first strand synthesis buffer. The temperature gradient profile used for cDNA synthesis was 5 min at 25°C, 60 min at 50°C, 15 min at 55°C, and 15 min at 75°C. The cDNA samples were diluted eight times to a final volume of 80 μl and stored at −20°C.

2.4 | Primer design

Nucleotide sequences of 12 selected maternal genes (beta-actin [*actb*], peptidylprolyl isomerase A [*ppia*], 5′-aminolevulinate synthase [*alas1*], succinate dehydrogenase complex flavoprotein subunit A [*sdha*], tyrosine 3-monooxygenase [*ywhae*], DND microRNA-mediated repression inhibitor 1 [*dnd*], DEAD-box helicase 4 [*vasa*], vegt protein [*vegt*], wingless-type MMTV integration site family member 11 [*wnt11*], DEAD-box helicase 25 [*ddx25*], orthodenticle homeobox 1 [*otx1*], and growth differentiation factor 1 [*gdf1*]) were identified in the de novo assembled sterlet transcriptome (Havelka, unpublished data). Gene search was conducted with BLAST. Reference nucleotide sequences of selected genes of *Xenopus leavis* and zebrafish *Danio rerio* (Hamilton, 1822) were downloaded from NCBI/Genbank database and subsequently searched against the de novo assembled transcriptome using the BLASTN algorithm. Sterlet nucleotide sequences were identified based on highest pairwise identity, highest bit score, and the lowest *e*-value. A panel of primers for amplification of selected maternal genes was designed with Primer3 (https://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 1). All primers were tested with RT-PCR using cDNA of unfertilized sterlet eggs and generated a single amplicon of the expected size on agarose gels.

2.5 | Quantitative PCR

The qPCR mix contained 2 μl of cDNA, 0.5 μl of the forward and reverse primers (10 μM), 5 μl of TATAA SYBR Grand Master Mix (TATAA Biocenter), and deionized water to a total volume of 10 μl. PCR was run in a real-time CFX384 cycler system (BioRad) with the cycling conditions of 30 sec at 95°C; and 45 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 15 sec, and elongation at 72°C for 10 sec. Melting curve was recorded from 65 to 95°C at 0.5°C intervals.

2.6 | Data analysis

The measured Cq values were converted to relative quantities assuming 100% PCR efficiency using the equation:

$$X_{section} = \frac{2^{(-Cqi)}}{\sum_{(j=1)}^{5} 2^{-Cqi}},$$

where Cq_i is the Cq value measured in the i^{th} section. The normalization was performed based on equal volumes of total RNA used for reverse transcription, essentially measuring gene-expression level relative to the total amount of RNA in each egg section. The transcript quantity in the sections was expressed as the fraction of the total number of mRNA molecules found in each of five segments along the animal–vegetal axis in the *A. ruthenus* oocyte. The relative quantities in each segment were averaged across all oocytes (Figure 1a, b).

3 | RESULTS AND DISCUSSION

The vegetal hemispheres store yolk, which is a strong inhibitor of biochemical reactions that can induce high variation in reverse transcription yield and compromise qPCR efficiency. All samples were tested for inhibition using an RNA spike (TATAA Biocenter) added to the extracted RNA, together with the random sequence hexamers and oligo-dT primers for reverse transcription. The low standard deviation of the RNA spike verified low variation in the RT and qPCR efficiencies (Figure 1a, b). Within this experiment with single cell—sturgeon oocyte—endogenous control could not be used due to lack of the gene which would be uniformly distributed within a cell.

The qPCR tomography revealed localization of selected genes in sturgeon oocyte (Figure 1a, b) and distinguished two groups of genes with significantly different expression profiles along animal–vegetal axis. The distribution of mRNAs coding germplasm determinants, including *dnd*, *vasa*, *ddx25*, and mesoderm inducer *gdf1*, showed an increasing gradient toward the vegetal pole, localized in the marginal segment and the vegetal hemisphere with highest abundance close to the pole. The high prevalence of mRNAs at the vegetal pole suggested that yolk in this region can also play a non-nutritional role. The animal hemisphere chiefly included reference genes, such as *actb*, *ppia*, *alas1*, *sdha*, and *ywhae*, but was also characterized by the expression of *vegt*, *wnt11*, and *otx1*. Notably, RNA profiles were highly conserved among individuals; there were no significant differences in expression level among corresponding segments of sturgeon oocytes.

This research was the first attempt to characterize RNA localization in sturgeon egg and to compare it with available patterns in the frog oocyte. We focused on markers that might be most reliably identified and compared with profiles in *Xenopus*, including markers of some of the most common reference genes, germplasm RNAs, and factors encoding specification of lineages in the oocyte. We constructed a fate

TABLE 1 Primers for RT-qPCR analysis

Gene	Function	Sequence 5′-3′	Amplicon length (bp)	Region
alas1	Encodes the mitochondrial enzyme which is catalyzes the rate-limiting step in heme biosynthesis	CCTACTTTTCTCCTCGTGCTTT AATTCATGGGCAACGTCG	294	105–381
actb	Encodes one of six different actin proteins that are involved in cell motility, structure and integrity	CACACAGTCCCCATCTACGAG CTGCCCCACGCCATCCTC	272	138–185
ppia	Encodes a member of the peptidyl-prolyl *cis-trans* isomerase (PPIase) family that accelerate the folding of proteins and may play role in immunosuppression	CTTCACCAACCACAACGGG ACAGGATCCCGGGGCCAG	105	127–214
sdha	Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase, a complex of the mitochondrial respiratory chain	GCAGGTTTGCCCAATCAA TCACGGATCTCAATGGTCATG	212	808–999
yawhz	This gene product belongs to the 14-3-3 family of proteins that are highly conserved and involved in many vital cellular processes such as metabolism, protein trafficking, signal transduction, apoptosis and cell cycle regulation	GAGGTTGCAGAAGGAGATGCT ACCCAAACGGATCGGATGT	117	277–375
vegt	The localized transcription factor, which operates sequentially in several developmental pathways during embryogenesis, including dorsoventral and posterior patterning of mesoderm	ACCGTATCCTTGCTGTCCAC GAAGGATGGCCTTTGTGAAA	211	501–711
otx 1	Encodes a member of the bicoid sub-family of homeodomain-containing transcription factors. The encoded protein acts as a transcription factor and may play a role in brain and sensory organ development.	CAGCAGCAACAGAGTGGAAA GGGTTGCTGATGGAGGTAGA	197	291–487
wnt11	Its ligands are expressed around the blastopore and play an important role in regulating cell movements associated with gastrulation	CAACGAGAACGACAAGCAAG ATCTCTCCACGGTCCTCTCA	203	18–220
dnd	Germline-specific protein required for the primordial germ cell (PGC) survival and migration during early embryonic development	GGACTCAGAAAATGGGGATCTCCCTGG AAACCTCACAGCCAGAGGAAGGGGG	108	141–248
vasa	This gene is specifically expressed in the germ cell lineage in both sexes and functions in germ cell development.	CAAGAATATCAGTAAATCGGGG GATCTGGTTTATTAGCTCTCTTGTT	244	587–806
vg1 (gdf1)	Encodes a member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues	AAGCCGATCACAATCCACTC GGTGTCTGTGTCCTGCTCAA	201	733–933
ddx25	Gonadotropin-regulated testicular RNA helicase is a germline specific RNA, found exclusively within the germplasm of oocytes and PGCs, as well as in male germ cells.	CTGACAATCGTTCGGTAGCA GAGCTCGCCTTACAGACAGG	291	498–788

map of germ layer specification and distribution of several gene groups along the animal–vegetal pole axis in *A. ruthenus* and *X. laevis* oocytes (Figure 2).

The resolution of five segments along the animal–vegetal axis of the sturgeon oocyte allowed identification of the distribution of maternal transcripts along the axis. We found clear similarity in the localization of mRNA molecules in *Acipenser ruthenus* and *Xenopus laevis*. Although the distribution of genes in oocytes shows a gradient character (Sindelka, Jonak, Hands, Bustin, & Kubista, 2008, Sindelka, Sidova, Svec, & Kubista, 2010), it is worth noting that the transition of genes in the sturgeon oocyte was slightly more regular than in *Xenopus*.

Based on expression patterns and their location in the egg, sturgeon genes were categorized as animal or vegetal, as reported in *Xenopus* (Sindelka et al., 2008). The group of animal genes included reference genes (*actb*, *ppia*, *alas1*, *sdha*, and *ywhae*), but also *vegt*, *wnt11*, and *otx1*, transcriptomes that have been observed in the vegetal hemisphere in *Xenopus* oocytes (Sindelka et al., 2010). The *vegt* gene is involved in the network pathway of endodermal specification during early embryogenesis in *Xenopus* (Clements et al., 1999). This pathway is partially conserved between *Xenopus* and zebrafish (Stainier, 2002). We hypothesize that *vegt*, which induces mesodermal differentiation in ectodermal regions in *Xenopus*, performs the same function in sturgeon, but with a slight shift to the animal region. The changes in morphological geometry during development might be due to the higher yolk volume in the sturgeon oocyte.

Maternal *wnt11* mRNA is also located in the frog oocyte vegetal cortex, and depletion of this gene results in disorders in dorsal–ventral patterning in the frog embryo (Elinson & Pasceri, 1989). In amphibian eggs, the plane of the first cleavage is perpendicular to the microtubules near the vegetal pole, and, in both amphibians and primitive

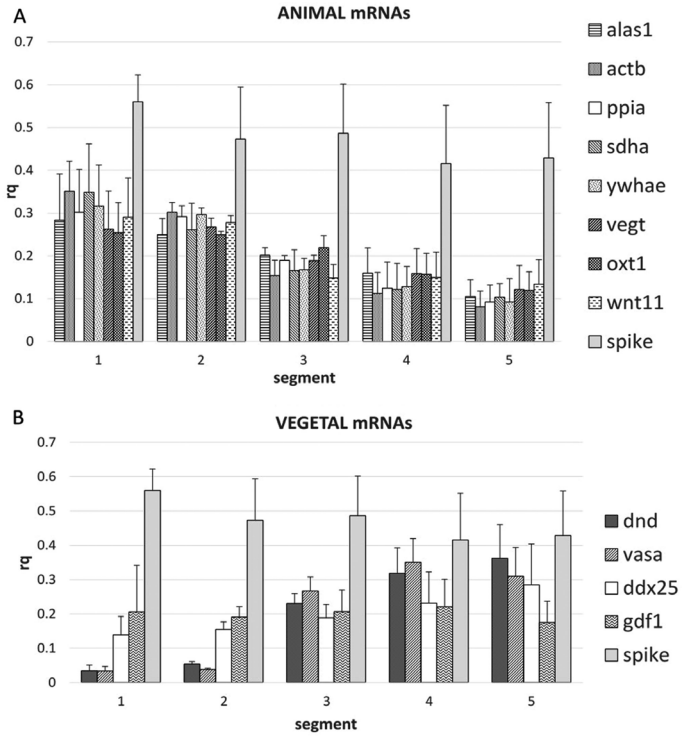

FIGURE 1 (A and B) Intracellular gradients of mRNA levels in *Acipenser ruthenus* oocytes. The distribution of animal pole genes (A) and vegetal pole genes (B) in the oocytes along the animal–vegetal axis. *Y*-axis indicates relative quantity and *X*-axis indicates the section from the animal pole (section 1) to the vegetal pole (section 5). The low standard deviation of the RNA spike verified low variation in the RT and qPCR efficiency. Error bars indicate standard error of the means

fishes, bisects the gray crescent along the meridian (Abraham, Miller, & Fluck, 1995). The wingless-type transcriptome in fertilized sturgeon eggs ensures cortical rotation, which results in formation of a gray crescent. In contrast to *Xenopus*, sturgeon embryo blastomeres cannot be separated into a dorsal and a ventral group, with equal number of animal and vegetal cells in each.

The *otx1* gene, a representative of the animal group in sturgeon, is involved in animal–vegetal orientation in *Xenopus* and is later expressed in developing head tissue (King et al., 2005). In vertebrates, *otx1* plays a role in early head specification involving interaction between the presumptive anterior neuroectoderm and mesendoderm during gastrulation (Mazan, Jaillard, Baratte, & Janvier, 2000).

A second group of transcriptomes included primarily germplasm markers, such as *dnd*, *vasa*, and *ddx25*, that could be defined as vegetal genes localized toward the extreme vegetal pole. Generally, localization of germplasm determinants in sturgeon was similar to profiles in *Xenopus*, thus demonstrating similarity of germ cell specification in sturgeon to that of anurans. We can conclude that localization of maternally derived germplasm determinants is characteristic of the preformation mode in both sturgeon and anurans.

The knowledge about localization of primordial germ cells can be used for identification of these cells in the oocytes with further utilization in variable bioengineering approaches in conservation or restoration of endangered fish species, induction of chimerism, or techniques linked to transplantation of germ cells and surrogate production. This is also indirectly an auxiliary tool in the development of techniques for sterilization.

The present study described localization of selected mRNAs in the sturgeon oocyte. Our observations corroborated previous work (Saito et al., 2006, 2014) showing that sturgeon PGCs are localized in the vegetal hemisphere close to the vegetal pole. Such similarities in expression profiles of distantly related species indicate that their ancestors could have arisen from more closely related lineages. The expression profiles of maternal genes in oocytes sturgeon and *Xenopus laevis* appear more similar than to those of other species in corresponding classes Actinopterygii and Amphibia.

The next step is to determine the role of the genes detected in the animal hemisphere of the sturgeon oocyte relative to their function in frog eggs, and to identify profiles of these genes during early developmental stages of sturgeon embryos. This will help determine if the

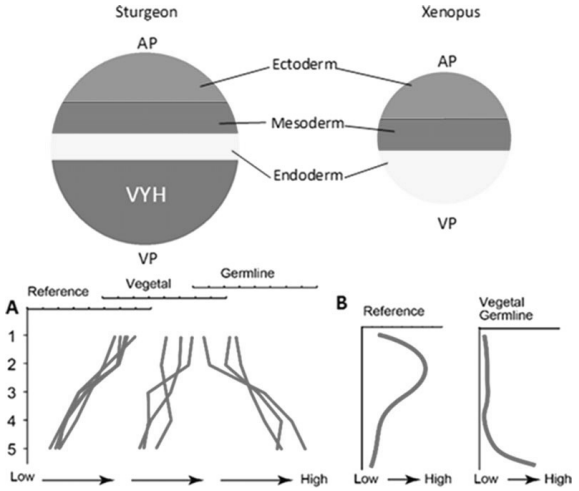

FIGURE 2 Fate maps showing germ layer specification and distribution of several gene groups along the animal–vegetal axis in *Acipenser ruthenus* and *Xenopus laevis* oocytes. The specification of germ layers prior to the axis (AP, animal pole; VP, vegetal pole): ectoderm (blue), mesoderm (red), and endoderm (yellow). In sturgeon, the germ layer origin shifted to the AP due to high lipid volume of vegetal yolky hemisphere (VYH). (a) Spatial distribution of genes in five segments of sturgeon oocyte along the animal–vegetal axis. (b) Spatial distribution of genes in Xenopus oocyte according to Sindelka et al., 2010 [Color figure can be viewed at wileyonlinelibrary.com]

vegetal yolky cells in sturgeon embryos are transcriptionally inactive, except for PGCs, and whether a recessive mechanism to suppress tissue differentiation exists, as well as characterize gene expression in the region. qPCR tomography is a promising technique to discriminate germ layers by altering cutting frequency.

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CHAPTER 3

THE TIMING AND CHARACTERIZATION OF MATERNAL TO ZYGOTE TRANSITION AND MID-BLASTULA TRANSITION IN STERLET *ACIPENSER RUTHENUS* AND *A. RUTHENUS* X *ACIPENSER GUELDENSTAEDTII* HYBRID

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RESEARCH PAPER

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The Timing and Characterization of Maternal to Zygote Transition and Mid-Blastula Transition in Sterlet *Acipenser ruthenus* and *A. ruthenus* x *Acipenser gueldenstaedtii* Hybrid

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Abstract

Detailed knowledge of early embryo development is a prerequisite for implementation of reproductive biotechnologies. We described the timing of mid-blastula transition in the sturgeon *Acipenser ruthenus* and hybrid of *A. ruthenus* and *Acipenser gueldenstaedtii*. To identify mitotic changes during cell division, the nuclei of embryo blastomeres were sampled every 30 min from 4 to 25 h post-fertilization and stained with 5 mg/ml 4'-6-diaminido-2-phenylindole. We observed embryos of hybrids to lose synchrony after the eighth cell division, one cycle earlier than in purebred sterlet embryos. To reveal the timing of zygotic gene activation, developing embryos were dechorionated and injected with 10, 50, 100, 250, or 500 µg/ml a-Amanitin. Termination of development in embryos was recorded throughout the period from the 1000 cell stage until gastrulation, showing the process of transition from maternal to zygotic gene transcription in sterlet to be of extended duration and not directly linked to mid-blastula transition.

Introduction

Mid-blastula transition (MBT) in early embryogenesis has traditionally been defined as a time point characterized by cell cycle lengthening, loss of synchrony, acquisition of cell motility, and onset of zygotic gene transcription (Signoret & Lefresne, 1971; Gerhart, 1980; Newport & Kirschner, 1982a). More recent studies treat maternal to zygotic transition (MZT) as a separate developmental period that begins with the elimination of maternal transcripts, continues through the production of zygotic transcripts, and concludes with the first major morphological requirement for zygotic transcripts in embryo development (Baroux, Autran, Gillmor, Grimanelli, & Grossniklaus, 2008;Tadros & Lipshitz, 2009). The MBT, as a point in MZT, has significance for development of complex physiological structures in the embryo. The initiation of zygotic transcription during MZT is well-established and evolutionarily conserved in all species (Tadros & Lipshitz, 2009; Langley, Smith, Stemple, & Harvey, 2014). Morphological changes during MBT represent milestones in the development of metazoans, since each cell transmits a perfect copy of its genome at each division.

The development of a pluripotent embryo to adult organism is consistent and strictly controlled at the cellular, as well as the genetic, level. The initial step in embryogenesis is the activation of egg upon fertilization, followed by the rapid division of the blastomeres. Prior to the early blastula stage, division occurs at a constant rate, with a reduced cell cycle that includes only the mitotic and short synthesis phases. Synchronous development of blastomeres in this period is achieved through this adjustment, with later inclusion of a gap phase, as well as by regulatory modification via mitosis-promoting factor inducing changes in the division rate (Newport & Kirschner, 1982a, Mauch & Schoenwolf, 2001). Analysis of DNA content of zebrafish *Danio rerio* cells in early stages have revealed that the G1 phase is transcription-dependent, and introduced into the

mitotic cycle only after launching of zygotic genes (Zamir, Kam, & Yarden, 1997). More recent studies have shown the G1 phase to be non-essential to deep cells, which form three germ layers in the embryo (Dalle Nogare, Pauerstein & Lane, 2009).

Desynchronization and cell cycle lengthening in fruit fly *Drosophila melanogaster* is reached at 14 cycles (Edgar, Kiehle, & Schubiger, 1986; Edgar & O'Farrell, 1990), in frog *Xenopus laevis* at 13 cycles (Newport & Kirschner, 1982a; Newport & Kirschner, 1982b; Masui and Wang, 1998), and in most teleost fish at 9 or10 cycles (Kane *et al.,* 1996a; Kane *et al*., 1996b; Yamaha, Mizuno, Matsushita, & Hasebe*,* 1999; Fujimoto *et al.,* 2004; Iwamatsu, 2004; Güralp, Pocherniaieva, Blecha, Policar, Pšenička, & Saito*,* 2016). In mammals and nematodes, the early mitotic divisions are asynchronous during cleavage, with the initial point of major zygotic transcript activation varying from the one cell stage to the four cell stage (Andeol, 1994; Tadros & Lipshitz, 2009). In some vertebrates, cell kinetics modifications occur nearly simultaneously with the elimination of maternal mRNA and subsequent transition to newly transcribed embryo genes (Newport & Kirschner, 1982a; Stroband, Krounie, & Gestel, 1992; Kane *et al*., 1996a, b). TATA-binding protein is required for establishment of zygotic gene expression and regulation and also participates in transcription initiated by RNA polymerases I, II, and III (Hernandez, 1993;Veenstra, Destrée, & Wolffe, 1999; Müller, Lakatos, Dantonel, Strähle, & Tora, 2001; Martianov, Viville, & Davidson, 2002). Maternal to zygotic reprogramming is characterized by degradation of maternal factors and activation of zygotic transcripts. The onset of zygotic genome activation (ZGA) is governed by an integrated model on several levels: histone modifications directly define chromatin status for transcription (Prioleau, Huet, Sentenac, & Méchali, 1994; Miao & Natarajan, 2005; Razin *et al*., 2007), increase in nucleus to cytoplasm ratio (Newport & Kirschner, 1982a; Kane & Kimmel, 1993), a developmental timer regulating degradation of Cyclin A and E1 proteins (Howe, Howell, Hunt, & Newport, 1995; Howe & Newport, 1996; Stack & Newport, 1997; Zegerman & Diffley, 2007), and lengthening of the cell cycle (Kimelman *et al*., 1987; Blythe & Wieschaus, 2015). Although the initiation of zygotic gene transcription is evolutionarily conserved, its mechanism is species-specific (Newport & Kirschner, 1982a; Kimelman, Kirschner, & Scherson, 1987; Almouzni & Wolffe, 1995; Neusser, Schubel, Koch, Cremer, & Müller, 2007).

The increase in nucleus to cytoplasm ratio has critical implications and is suggested to be a primary factor in MBT induction (Newport & Kirschner, 1982a; Kane & Kimmel, 1993), as demonstrated by experiments with mechanical constriction of the cytoplasm, induction of polyspermy, and injections of exogenous nonspecific DNA (Newport & Kirschner, 1982a; Etkin, 1988; Kane & Kimmel, 1993; Lee, Bonneau, & Giraldez, 2014). Mid-blastula transition cannot be viewed as a single transition, but as multiple independent processes that occur during early development (Gerhart, 1980; Yasuda & Schubiger, 1992; Langley, Smith, Stemple, & Harvey, 2014).

Bio-engineering consists of micromanipulation of cells of undetermined fate (Fujimoto *et al*., 2004). The cell specification in early development leads to motility, proliferation, and adhesiveness of cells before germ layer formation (Gerhart, 1980). Bio-techniques can be employed in artificial reproduction. To increase success of techniques based on cell manipulation and transplantation, knowledge of changes and timing of such processes is essential be determined.

The goal of this study was to determine the timing of zygotic gene activation and to characterize MBT in *Acipenser ruthenus*, the population of which has sharply declined due to anthropological activity.

Materials and Methods

Ethics Statement

Experimental procedures were carried out in accordance with Czech Law 246/1992 on animal welfare, for which the authors possess a certificate according to §17 of the law. Protocols underwent ethics review and experimental activities were conducted in facility having authorization for the use of experimental animals No.: 53100/2013-MZE-17214 valid from 08/30/2013 to 08/30/2016.

Fish

Oocytes of sterlet *Acipenser ruthenus* and crosses of *A. ruthenus* and Russian sturgeon *Acipenser gueldenstaedtii* were used. *Acipenser ruthenus* and *A. gueldenstaedtii* have different ploidy levels produces differences of DNA content in their hybrid offspring (Birstein, Poletaev, & Goncharov, 1993), making the embryo a useful model for investigation, due to changes in the timing of early development. Ovulation was induced in sterlet *A. ruthenus* with intramuscular injections of carp pituitary extract powder dissolved in 0.9% (w/v) NaCl solution at an initial dose of 0.5 mg/kg of body weight, followed by a second injection of 4.5 mg/kg of body weight 12 h after the first injection. The oocytes were collected from five fish via a minimally invasive incision of the oviduct 18–20 h after the second injection. To induce spermiation, males received an intramuscular injection of carp pituitary extract powder dissolved in 0.9% (w/v) NaCl solution at a single dose at 4.0 mg/kg of body weight. Sperm was collected 48 h post-injection from the urogenital papilla using a catheter, transferred to a separate cell culture container (250 ml), and stored at 4°C until use. Eggs were fertilized with sperm activated in dechlorinated tap water at 15°C. Stickiness of the fertilized eggs was removed by treating with 0.1% tannic acid solution. Determination of the early developmental stages was based on studies of *A.*

gueldenstaedtii, *A. stellatus*, *Huso huso*, *and Acipenser baerii* (Ginsburg & Dettlaff, 1991; Park, Lee, Kim, & Nam, 2013), as their developmental pattern is similar to that of sterlet (Bolker, 2015). Embryos were maintained in dechlorinated tap water at 18°C to the desired stage (neurulation) for 28-32 h.

Nucleus Visualization with 4'-6-Diaminido-2-Phenylindole (DAPI) Staining

The eggs from four sterlet females were fertilized individually with the sperm from four Russian sturgeon males – eggs of the first two females were fertilized by mix of sperm of the first two males, while eggs of next two females were fertilized by mix of sperm of the next two males. As a control were used eggs from three sterlet females and sperm from three sterlet males, eggs from each female were fertilized individually by mix of sperm from three males. Ten embryos from four hybrid groups (sterlet female x Russian sturgeon male) and from three control sterlet groups were selected every 30 min from 4 to 25 h post-fertilization (hpf), fixed in 2% glutaraldehyde in phosphate-buffered saline, and stored at 4°C. Samples were dehydrated in pure methanol and washed in Tris-buffered saline (TBS) containing 0.1–0.25% Triton™ X-100 (Sigma-Aldrich). Thin sections were cut of the animal hemisphere, including the marginal zone, using a sterile stainless steel No.10 scalpel blade (Swann-Morton, England). Nuclei of sterlet and hybrid embryonic cells were stained with 5 mg/ml DAPI dissolved in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.15 M NaCl for 5 min. Blastomeres were washed in TBS with glycerol and observed under an inverted fluorescence microscope (Olympus Model IX83).

Transcription Inhibition

For transcription-blocking, we used α-Amanitin (A2263), a bicyclic octapeptide that inhibits eukaryotic RNA polymerase II and III (Stirpe & Fiume, 1967). Eggs from ten sterlet females and sperm from six sterlet males were used for fertilization. Eggs from the first five females were fertilized by combination of sperm from the first three males and eggs from the next five females were fertilized by combination of sperm from the next three males. Fertilized sterlet eggs were dechorionated using forceps and injected at the animal pole with 10, 50, 100, 250, or 500 µg/ml α-Amanitin. Embryos injected with nuclease-free water were used as control (n = 60 per group). An aqueous stock solution of 1 mg/ml α-Amanitin was diluted in nuclease-free water with 0.2-M KCl immediately prior to use. A glass microcapillary was drawn from a glass needle (Drummond, Japan) using a needle puller (PC-10; Narishige, Japan). Microinjection was performed in agarose gel under a stereomicroscope (Nikon, Japan) using a micromanipulator M-152 (Narishige, Japan) and FemtoJet express microinjector (Eppendorf, Germany) with a pressure of 100 hPa for 1 sec.

Evaluation and Data Analysis

Mortality was assessed visually based on development, colour, and structure of the embryo. Mean survival rate of the five treated groups and controls at various stages of development. Differences in survival rate were assessed based on the standard deviation implemented in Microsoft Excel program.

Synchronous cleavage before MBT was characterized by two phases of the cell cycle: the cell mitotic phase (prophase, metaphase, anaphase and telophase) and the synthesis phase. To determine this status we counted the number of nuclei at the same phase at each time point of fixation. The determination of asynchrony was based on the detection of three or more phases of the cell cycle.

Results

Morphological Changes

To characterize changes in cell cycle patterns during early developmental stages, we visualized nuclei of blastomeres in the animal pole using DAPI staining. The cell nuclei in the sections of animal hemisphere, including the marginal zone, were examined to identify mitotic phases. The identification of two adjacent phases was considered synchronous division, since embryos could be fixed at the time of transition to the next mitotic phase. Cells divided synchronously at a constant rate until MBT at the ninth cell cycle in sterlet embryos that corresponds to 1000 cell stage (13 hpf) (Figure 1A). The sterlet x Russian sturgeon hybrid embryos showed transition from synchronous to asynchronous division at the eighth cell cycle which is the 512 cells stage (12 hpf). Asynchrony was determined as simultaneous appearance of nuclei at three different mitotic phases: pro-, meta- and telo-phase (Figure 1B). In both sterlet and hybrid embryos, the transition occurred within 1 h.

Inhibition of Sterlet Zygotic Genes

No effect on cell developmental pattern during either early or late development was detected. At 2 to 1000 cells, treated embryos were indistinguishable from siblings injected with water and non-injected controls embryos. Injected embryos developed normally during the cleavage and early blastula periods, as expected, due to little or no transcription before MBT. The earliest effect of the α-Amanitin injection was cessation in cell division at the 1000 cell stage (14 hpf), 1 h after onset of MBT (Figure 2), seen in a mean of 65% of embryos at all α-Amanitin concentrations. After the tenth cleavage during late blastula, when blastomeres in the animal pole are surpassed 1000 cells, the treated embryos began to die. The highest embryo mortality was observed after reaching 1000 cell stage - 33.3 %, and most of the remaining injected embryos died

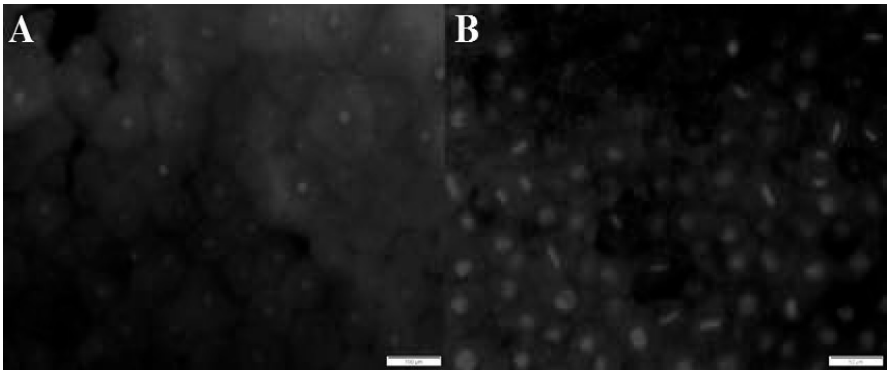

Figure 1. Fluorescence micrographs of cleavage cycle in *Acipenser ruthenus* embryos. (A) Synchronous S phase in majority of nucleus in the 9th cell cycle (13 hpf), scale bar = 100 µm. (B) Asynchronous mitotic phases represented by three different phases (pro-, meta- and telophase) of nuclei in blastomeres in the 10th cell cycle (14 hpf), scale bar = 50 µm.

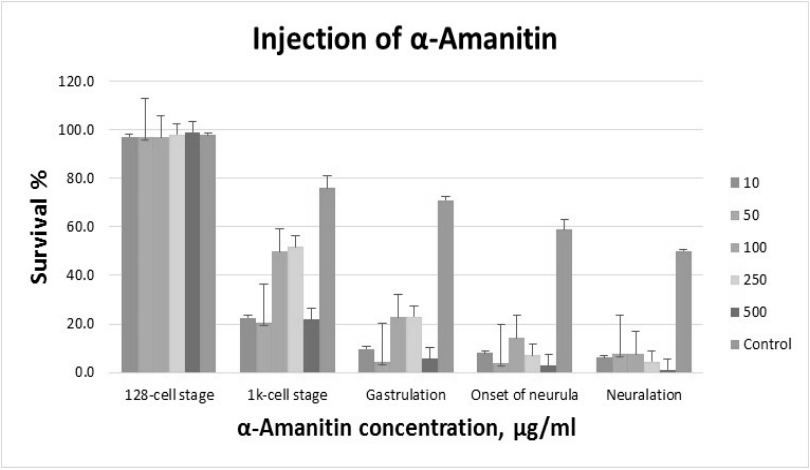

Figure 2. Survival of *Acipenser ruthenus* embryos during development after injection with α-Amanitin. Error bars indicate mean ± standard deviation of results from serial experiments.

subsequently. There was no correlation between embryo survival and concentration of α-Amanitin ($P<0.05$). A small proportion of α-Amanitin-injected embryos went through gastrulation, neurulation, and reached hatching (data not shown).

Discussions

We identified the transition period from synchronous to asynchronous cell division in sterlet embryos and hybrid crosses by visualization of cell nuclei with DAPI, referred to as initial MBT. And using α-Amanitin treatment we determined the initiation of zygotic genome activation in sterlet embryos.

The subsequent morphogenetic aspect of embryogenesis includes the onset of cell movement. The period preceding gastrulation, characterized by dramatic changes in embryo development, has been described in several teleost fish species: zebrafish *Danio rerio* (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995), goldfish *Carassius auratus* (Yamaha, Mizuno, Matsushita, & Hasebe, 1999), medaka *Oryzias latipes*

(Iwamatsu, 2004), loach *Misgurnus anguillicaudatus* (Fujimoto et al., 2004), pikeperch *Sander lucioperca* (Güralp, Pocherniaieva, Blecha, Policar, Pšenička, & Saito, 2016). We found sterlet embryos to undergo nine cell cycles before the onset of desynchronization, with complete asynchrony in the tenth cycle, similar to zebrafish and goldfish (Kane & Kimmel, 1993; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Yamaha, Mizuno, Matsushita, & Hasebe, 1999). The comparison of our results with fluorescent micrographs of cleavage cycle in embryos of loach (Fujimoto et al., 2004) and pikeperch (Güralp, Pocherniaieva, Blecha, Policar, Pšenička, & Saito, 2016) shows clear difference in timing of synchronous to asynchronous transition. Medaka, loach and pikeperch required ten synchronous cycles which is correspond to the 1000 cell stage which is one cell cycle later than in sturgeon. The main sources of desynchronization are presumed to be the G1 and G2 phases, as well as a direct extension of the M and S phases (Newport & Kirschner, 1982a; Edgar, Kiehle, & Schubiger, 1986; Kane & Kimmel, 1993). The desynchronization in sturgeon and frog is a temperature-dependent event, which can easily be shifted by changing the temperature of the water (Chulitskaia, 1970). It is possible to delay or accelerate embryo development and the period of transition, but only within the allowable temperature range for the species. For the present study, we used 18 ºC and the he appropriate temperature range for incubation of sturgeon eggs is from 10 to 25 ºC (Ginsburg & Dettlaff, 1991; Dettlaff, Ginsburg, & Schmalhausen, 1993).

Early research on MBT showed that zebrafish and *Xenopus* are similar in mode of MBT, which includes simultaneous cell cycle lengthening and onset of zygotic gene transcription (Newport & Kirschner, 1982a; Edgar, Kiehle, & Schubiger, 1986; Kane & Kimmel, 1993; Masui & Wang, 1998). Nevertheless, MBT and ZGA are relatively independent of each other, even in timing, and based on different mechanisms, as introduction of the new phase in cell cycling is a transcriptionally independent event and does not play a direct role in ZGA initiation (Dalle Nogare, Pauerstein, & Lane, 2009; Zhang et al., 2014).

Hybridization of sterlet and Russian sturgeon leads to an increase in DNA content (Birstein, Poletaev, & Goncharov, 1993), and these embryos reach the threshold of the nucleocytoplasmic ratio one cycle sooner. In zebrafish, cell cycle lengthening occurs one cleavage later in haploid embryos compared with normal diploid embryos, and one cleavage earlier in tetraploid embryos (Kane & Kimmel, 1993). This is also confirmed by induction of polyspermy or injecting plasmid DNA, which leads to early transcription, since it reduces compounds capable of suppressing DNA (Newport & Kirschner, 1982a; Prioleau, Huet, Sentenac, & Méchali, 1994). Thus, it can be assumed that MBT in sturgeon, as well as in zebrafish and *Xenopus*, is regulated by the ratio of nucleus to cytoplasm.

To reveal MZT in sterlet, we used transcription inhibitor α-Amanitin. We aimed to clarify the temporal connection of onset of zygotic gene transcription with desynchronization of the cell cycle. The concentrations of α-Amanitin used in this experiment were similar to the concentration that arrests RNA polymerase II in carp *Cyprinus carpio* (Stevens, Schipper, Samallo, Stroband, & Kronnie, 1998), *Xenopus* (Newport & Kirschner, 1982a) and *Drosophila* (Edgar, Kiehle, & Schubiger, 1986), as well as the concentration that causes an detectable effect in zebrafish - an arrest of development 4.5 h after fertilization (Kane et al., 1996a). Embryos of zebrafish treated with α -amanitin passed through the MBT retaining the typical shape for that stage and began to die at 8 to 10 hpf (Kane et al., 1996a). In the experiment on carp embryos blocking of epiboly 6 h after fertilization had been shown in presence of α-Amanitin and maintained a late-blastula like shape until degenerating after 10 h of development, although cleavage and formation of layers were not affected by the α-Amanitin (Stroband, Krounie, & Gestel, 1992; Stevens, Schipper, Samallo, Stroband, & Kronnie, 1998). Embryos of sturgeon injected with α-Amanitin also showed cell cycle kinetics similar to controls, with no delay or malformation during cleavage. We identified the point of termination in embryonic development, which was presumably due to inhibition of proper function of newly transcribed genes. Further development to gastrulation by the surviving embryos can be explained as a delay in degradation of maternal mRNAs and interruption of zygotic gene expression. Transcription blocking showed MBT to include only desynchronization and lengthening of the cell cycle with no direct link to the switch to transcription of parental genes.

The significant characteristic of the most fish species is the meroblastic cleavage pattern of their eggs, while sturgeon present a holoblastic pattern and several features similar to *Xenopus* (Macgregor, 1972; Elinson, 2009; Saito et al., 2014, Pocherniaieva, Sidova, Havelka, Saito, Psenicka, & Kaspar, unpublished data). The ability of eggs to be activated without fertilization is a characteristic of *Acipenseridae*. Parthenogenesis in sturgeon embryos after egg activation leads to irregular cleavage with arrest in development, although mechanism of this process remains unclear (Dettlaff, Ginsburg, & Schmalhausen, 1993). In unfertilized eggs, the onset of MZT immediately after egg activation with elimination of the maternal products in the egg (Tadros & Lipshitz, 2009) eventually results in degradation of mRNAs and proteins with subsequent death of the embryo. Hence, we can hypothesize that the first step of MZT, destabilization in the network of maternal factors, occurs before late blastula, since all sturgeon embryos undergo eight cleavages after activation (Dettlaff, Ginsburg, & Schmalhausen,1993).

Zygotic gene activation can be triggered by increase of DNA content in sturgeon through

polyploidization, which is a common phenomenon in these genera (Birstein, Poletaev, & Goncharov, 1993). This feature can be used in further research to investigate ZGA in sturgeon and clarify steps in maternal to zygotic transition.

A goal in fishery and aquaculture is to increase production via biotechnological innovations, as well as application of techniques that can eliminate the negative effects of human activity on natural fish populations. A variety of gene-manipulation techniques, including selection, intraspecific crossbreeding, interspecific hybridization, sex reversal, and polyploidy, have been commercially implemented to improve the culture of fish and shellfish (Dunham, 2004). The knockout and / or knockdown of a germplasm genes, such as dead end (dnd 1) for elimination of germ cells is essential for surrogate production of sturgeon species included in the IUCN list. Utilization of these methods requires a solid background in basic developmental processes of the target organism. Our results may have significant implications for biotechnological approaches such as blastomere transplantation, nuclear transfer, and microinjection techniques, since it reveals the timing of MBT and of ZGA.

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CHAPTER 4

General discussion

The beginning of the new century has been marked in the developmental biology by a keen interest in studying the phenomenon of the impact of maternal factors with an emphasis on their localization (Martin and Ephrussi, 2009).

Maternal factors play a key role in early development and control of all processes in the newly-formed organism before the activation of the embryonic genome (Houston, 2013). Moreover, the localization of maternal factors in the certain areas of the oocyte body plan is a highly conserved strategy, which regulates early embryonic development (Blanpain et al., 2004; Knoblich, 2008).

The molecular mechanisms by which maternal determinants regulate embryonic development and their spatial distribution in the oocyte of the ancient fish remain unclear. The main source of maternal products are mRNA and proteins that accumulate in the oocyte during oogenesis. Here we focused on localization patterns of several maternal mRNAs in sturgeon egg and on degradation of maternal stock with the switch to zygotic genes in the embryo. And thus, show the way from one cell - egg, which development completely relied on the maternal contribution to an independent unit capable to maintain processes essential for the functioning of the whole organism.

One of the important goals of fisheries and aquaculture in recent decades has been to increase production through biotechnological innovations, as well as the application of methods that can at least partially treat the negative effects of human activity on natural fish populations. A variety of breeding strategies, including selection, intraspecific crossbreeding, interspecific hybridization, sex reversal, and polyploidy, have been commercially implemented to improve the culture of fish and shellfish (Dunham, 2004). Important aspect of our study rely on localization of primordial germ cells that can be used for the further utilization in variable bio-engineering approaches in conservation or restoration of endangered fish species, induction of chimerism or techniques linked to transplantation of germ cells and surrogate production. Surrogate production is believed to be a promising approach for the conservation of rare and endangered fish species and the knowledge of all aspects of development of embryo, including an understanding of PGSc specification,and detailed knowledge of MZT may have significant impact on its successful utilization. This knowledge is also indirect an auxiliary tool in the development of techniques for sterilization. Since, utilization knockout and / or knockdown techniques for surrogate production of endangered sturgeon species requires a solid background in basic developmental processes of the target organism. Moreover, the results of our study show the time of the mid-blastula transition (MBT), as well as the timing of zygotic gene activation (ZGA) and can be significant in improving the methods of blastomere transplantation, nuclear transfer, and microinjection, since they indicate the time period when the embryo manipulation should be completed.

The research described in Chapter 2 was the first attempt to demonstrate RNA localization in sturgeon egg and to compare it with available patterns in the frog oocyte. Basically, dynamic of oogenesis can vary considerably in different species, but accumulation of highly active substances, such as cytoplasmic enzymes, organelle, mRNAs, proteins and metabolic substrates in the egg is common. All these components are distributed in oocytes of sturgeon and frogs along animal-vegetal axis. This distribution of maternal mRNAs exhibited in Chapter 2 was obtained by method of qPCR tomography developed by Sindelka et al. (2008). This technique has shown its effectiveness and relative easyness by cutting eggs with the dense yolk for sections along its animal-vegetal axis. Subsequently, mRNA levels of egg slices were measured using real-time RT-PCR analysis. In our research we focused on markers that might be most reliably identified and compared with already existing profiles in *Xenopus*. And also

markers of some of the most common reference genes, germplasm RNAs, and factors encoding specification of lineages in the oocyte. Thereafter, we constructed a fate map of germ layer specification and distribution of several gene groups along the animal-vegetal pole axis in *A. ruthenus* and *X. laevis* oocytes. Maternal determinants subdivided for several clusters according to their distribution in the sturgeon ooplasm showing the assymetrical localization along animal-vegetal axis. The distribution of mRNAs coding germplasm determinants, including *dnd*, *vasa*, *ddx25*, and mesoderm inducer *gdf1*, showed an increasing gradient toward the vegetal pole, localized in the marginal segment and the vegetal hemisphere with highest abundance close to the pole. Thus, we have proved that the high prevalence of mRNAs at the vegetal pole suggested that yolk in this region can also play a non-nutritional role. The localization of selected mRNAs observed in the sturgeon oocyte corroborated previous work (Saito et al., 2006, Saito et al., 2014) showing that sturgeon PGCs are localized in the vegetal hemisphere close to the vegetal pole. Thus, the data obtained in Chapter 2 together with the previous research results of our colleagues indicates that the vegetal part of the sturgeons´ egg is where the germ cells originate.

The animal hemisphere mostly included reference genes, such as *actb*, *ppia*, *alas1*, *sdha*, and *ywhae*, which can be explained by proximity to the nucleus to ensure passive transport of transcripts from the nucleus. Also *vegt*, *wnt11*, and *otx1* were identified in the animal hemisphere. We suggested that these determinants have a different localization compared to *Xenopus* due to some features in the structure and content of the sturgeon oocyte. The *vegt*, which induces mesodermal differentiation in ectodermal regions in *Xenopus*, performs the same function in sturgeon, but with a slight shift to the animal region due to the higher yolk volume in the sturgeon oocyte. The *otx1* gene, a representative of the animal group in sturgeon, is involved in animal-vegetal orientation in *Xenopus* and is later expressed in developing head tissue (King et al., 2005). In vertebrates, *otx1* plays a role in early head specification involving interaction between the presumptive anterior neuroectoderm and mesendoderm during gastrulation (Mazan et al., 2000). Maternal *wnt11* mRNA is also located in the frog oocyte vegetal cortex, and depletion of this gene results in disorders in dorsal-ventral patterning in the frog embryo (Elinson and Pasceri, 1989). In contrast to *Xenopus*, sturgeon embryo blastomeres cannot be separated into a dorsal and a ventral group, with equal number of animal and vegetal cells in each. Thus, we hypothised the wingless-type transcriptome (*wnt11*) in fertilized sturgeon eggs can ensures cortical rotation, which results in formation of a gray crescent.

Generally, the localization of selected mRNA molecules has the similar character in *Acipenser ruthenus* and *Xenopus laevis*. The distribution of genes in oocytes has a gradient character, which is determined by various types of transport paths implemented in the oocyte (Sidova et al., 2015). Moreover, the localization of maternally derived germplasm determinants is characteristic of the preformation mode in both sturgeon and anurans.

The qPCR tomography is a promising technique to determine the role of the genes detected in body plan of the sturgeon oocyte, and to identify profiles of these genes during early developmental stages of sturgeon embryos (Sindelka et al., 2008). This will help to determine if the vegetal yolky cells in sturgeon embryos are transcriptionally inactive, except for PGCs, and whether a recessive mechanism to suppress tissue differentiation exists, as well as characterize gene expression and their pathways in the region. The real-time RT-PCR tomography would also be applied to track migration of PGCs, as well as identify the transcription of germ cells genes involved in the specification and differentiation of cells. In addition, it can be a reliable tool for assessing the success and efficiency of donor cell transfer to the recipient's body in various transplantation and cloning methods. Or it can also be used to localize viruses and bacteria in tissue sections (Sindelka et al., 2008).

In third chapter of the thesis, we attempt to clarify the temporal connection of onset of zygotic gene transcription with desynchronization of the cell cycle. We identified the transition period from synchronous to asynchronous cell division in sterlet embryos and hybrid crosses by visualization of cell nuclei with DAPI, referred as initial mid-blastula transition. By application of α-Amanitin treatment we determined the initiation of zygotic genome activation in sterlet embryos. α-Amanitin is a highly toxic cyclic octopeptide found in genus of mushrooms known as Amanita, including *Amanita phalloides, Amanita verna*, and *Amanita virosa*. The cytotoxicity found in amanitin is the result of inhibition of RNA polymerases, in particular RNA polymerase II, which precludes mRNA synthesis (Lindell et al., 1970). The concentrations of α-Amanitin used in this experiment were similar to the concentration that arrests RNA polymerase II in carp (Stevens et al., 1998), frog (Newport and Kirschner, 1982a) and Drosophila (Edgar et al. 1986), as well as the concentration that causes an detectable effect in zebrafish - an arrest of development in early epiboly, 4.5 h after fertilization. Embryos of zebrafish treated with α -amanitin passed through the MBT retaining the typical shape for that stage and began to die in late epiboly, 8 to 10 hpf (Kane et al., 1996a). In the experiment on carp embryos blocking of epiboly 6 h after fertilization had been shown in presence of α-Amanitin and maintained a late-blastula like shape until degenerating after 10 h of development, although cleavage and formation of layers were not affected by the α-Amanitin (Stroband et al., 1992; Stevens et al., 1998). In our study embryos of sturgeon injected with α-Amanitin also showed cell cycle kinetics similar to controls, with no delay or malformation during cleavage. We identified the point of termination in embryonic development, based on inhibition of proper function of newly transcribed genes. Further development to gastrulation by the surviving embryos can be explained as a delay in degradation of maternal mRNAs and interrupted zygotic gene expression. The period preceding gastrulation, characterized by dramatic changes in embryo development, has been described in several teleost fish species: zebrafish *Danio rerio* (Kane and Kimmel, 1993, Kimmel et al., 1995), goldfish *Carassius auratus* (Yamaha et al., 1999), medaka Oryzias latipes (Iwamatsu, 2004), loach *Misgurnus anguillicaudatus* (Fujimoto et al., 2004), pikeperch *Sander lucioperca* (Güralp et al., 2016).

It is possible to delay or accelerate embryo development and the period of transition, but only within the allowable temperature range for the species. In our experiment, we used 18 ºC and the appropriate temperature range for incubation of sturgeon eggs is from 10 to 25 ºC (Ginsburg and Dettlaff, 1991; Dettlaff et al., 1993). We reported that sterlet embryos undergo nine cell cycles before the onset of desynchronization, with complete asynchrony in the tenth cycle, similarly to zebrafish and goldfish (Kane and Kimmel, 1993; Kimmel et al., 1995; Yamaha et al., 1999). The comparison of our results with fluorescent micrographs of cleavage cycle in embryos of loach (Fujimoto et al., 2004) and pikeperch (Guralp et al., 2016) shows clear difference in timing of synchronous to asynchronous transition. Medaka, loach and pikeperch required ten synchronous cycles which is corresponding to the 1000 cell stage comming one cell cycle later than in sturgeon. The main sources of desynchronization are presumed to be the G1 and G2 phases, as well as a direct extension of the M and S phases (Newport and Kirschner, 1982a; Edgar et al., 1986; Kane and Kimmel, 1993). The desynchronization in sturgeon and frog, as organisms with external egg development, is a temperature-dependent event, which can easily be shifted by changing the temperature of the water (Chulitskaia, 1970). Moreover, the MBT in sturgeon, as well as in teleost fish and anurans, is controlled by the ratio of nucleus to cytoplasm, which can subsequently be controlled using hybridization, induction of polyspermy or injecting plasmid DNA (Newport and Kirschner, 1982a; Prioleau et al., 1994). It has been shown in zebrafish that cell cycle lengthening occurs one cleavage later in haploid embryos compared with normal diploid embryos and one cleavage earlier in tetraploid embryos (Kane and Kimmel, 1993). This has to be taken into account that Acipenseridae is

the only group among vertebrates all members of which can hybridize with each other in the wild if their spawning grounds overlap, even despite the incompatibility of their genomes (Arnold, 1997). Based on cytogenetic data all Acipenseriformes species can be divided into three groups: first group with approximately 120 chromosomes and 3.2–4.6 picograms (pg) of DNA; second group with approx. 250–270 chromosomes and 6.1–9.6 pg of DNA; and single species, the shortnose sturgeon *Acipenser brevirostrum* with 13.1 pg of DNA (Blacklidge and Bidwell, 1993) and a chromosome number of around 360 (Kim et al., 2005). The hybridization of sterlet from the first group with ±120 chromosomes and Russian sturgeon from second group with ±250 chromosomes leads to an increase in DNA content (Birstein et al., 1993) and these hybrid embryos have reached the threshold of the nucleocytoplasmic ratio one cycle sooner in our experiment. Thus, time of activation of the zygotic gene can be changed in sturgeons through polyploidization or hybridization.

The next special feature of the Acipenseriformes is the ability of eggs to be activated without fertilization. Parthenogenesis in sturgeon embryos after egg activation leads to irregular cleavage with arrest in development, although mechanism of this process remains unclear (Dettlaff et al., 1993). In unfertilized eggs, the onset of MZT immediately after egg activation with elimination of the maternal products in the egg (Tadros and Lipshitz, 2009) eventually results in degradation of mRNAs and proteins with subsequent death of the embryo. Hence, we hypothesized that the first step of MZT, destabilization in the network of maternal factors, occurs before late blastula, since all sturgeon embryos undergo eight cleavages after activation (Dettlaff et al., 1993).

The significant characteristic of the most fish species is the meroblastic cleavage pattern of their eggs, while sturgeon present a holoblastic pattern and several features similar to *Xenopus* (Macgregor, 1972; Elinson, 2009; Saito et al., 2014; Pocherniaieva et al., 2018). Early research on MBT showed that zebrafish and Xenopus are similar in mode of MBT, which includes simultaneous cell cycle lengthening and onset of zygotic gene transcription (Newport and Kirschner, 1982a; Edgar et al., 1986; Kane and Kimmel, 1993; Masui and Wang, 1998). Nevertheless, MBT and ZGA are relatively independent of each other, even in timing, and based on different mechanisms, as introduction of the new phase in cell cycling is a transcriptionally independent event and does not play a direct role in ZGA initiation (Dalle Nogare et al., 2009; Zhang et al., 2014). Transcription blocking showed MBT to include only desynchronization and lengthening of the cell cycle with no direct link to the switch to transcription of parental genes.

In conclusion, I believe that our studies related to intracellular profiling in sturgeon eggs, together with the fundamental knowledge about the processes occurring during sturgeon embryogenesis, will help in the successful application and improvement of various modern molecular and biotechnological approaches.

Conclusion:

1. We identified the distribution of two major groups of maternal mRNAs with significantly different expression profiles in the ooplasm along the animal-vegetal axis of the sturgeon oocyte.

2. The maternally synthesized mRNAs showed different gradients inside the vegetal group: vegetal gradient - with high prevalence in the marginal segment and vegetal hemisphere and the extreme vegetal gradient - with highest abundance in vegetal pole close to the cortex.

3. The localization of mRNAs´ coding germplasm determinants showed an increasing gradient toward the vegetal pole. The distribution of maternally derived germplasm determinants is characteristic of the preformation mode in both sturgeon and anurans.

4. The clear similarity in the localization of mRNA molecules in such distinguished organisms as *Acipenser ruthenus* and *Xenopus laevis* was found.

5. The initiation of zygotic genome activation in sterlet embryos was determined in tenth cleavage during late blastula.

6. We identified initial mid-blastula transition in sterlet embryos and hybrid crosses of Russian sturgeon *A. gueldenstaedtii* x sterlet *A. ruthenus*, which is characterized the transition period from synchronous to asynchronous cell division.

7. It was shown that mid-blastula transition has no direct link to the onset of *de novo* transcription from zygotic genome and include only desynchronization and lengthening of the cell cycle.

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English summary

The foundation of maternal factors in sturgeon: from oocyte to embryo

Kseniia Pocherniaieva

A decrease in wild sturgeon populations in recent decades has led to growing interest in the use of variety of bio-engineering approaches in conservation or restoration of endangered fish species. Induction of chimerism, germ cells transplantation or surrogate production are methods that might lead to restoration of the fish stocks and these methods are promising for conservation purposes. The effective application of such embryo engineering to endangered sturgeon species requires fundamental knowledge of its embryonic development and information about structure and characteristics of sturgeon oocyte itself. To reveal intracellular geometry, mechanisms of maternal determinants organization and its later reorganization and morphogenetic aspects we used several techniques such as qPCR tomography, inhibition of transcription and visualization of nucleuos.

The qPCR tomography was discovered as reliable technique to determine the role of the genes detected in the animal and vegetal hemispheres of the sturgeon oocyte, and to identify profiles of these genes during early developmental stages of sturgeon embryos. The qPCR tomography was basically performed in two steps: RT-PCR itself and preliminary steps of the tomography. The optimally oriented oocytes were cut into 30 µm sections, across the animal-vegetal axis for subsequent total RNA extraction. The yolk presented in sturgeon eggs also plays a role of the main inhibitor of the PCR reaction.

The 12 selected maternal genes [beta-actin (*actb*), peptidylprolyl isomerase A (*ppia*), 5′-aminolevulinate synthase (*alas1*), succinate dehydrogenase complex flavoprotein subunit A (*sdha*), tyrosine 3-monooxygenase (*ywhae*), DND microRNA-mediated repression inhibitor 1 (*dnd*), DEAD-box helicase 4 (*vasa*), vegt protein (*vegt*), wingless-type MMTV integration site family member 11 (*wnt11*), DEAD-box helicase 25 (*ddx25*), orthodenticle homeobox 1 (*otx1*), *and* growth differentiation factor 1 (*gdf1*)] were investigated.Two groups of transcriptomes categorized as animal or vegetal with evident gradient profile were identified. The primarily germplasm markers such as *dnd*, *vasa*, *ddx25* were localized toward the extreme vegetal pole. This finding reveals localization of primordial germ cells in the body plan of the sturgeon oocyte.

Another aspect of applying such technique was comparative analysis of RNA profiles in the oocyte of distantly-related species *Xenopus laevis* and *Acipenser ruthenus*. We found clear similarity in the localization of mRNA molecules in *Acipenser ruthenus* and *Xenopus laevis*, which revealed significant aspects of early development that have been conserved during evolution. Such similarities in expression profiles of distantly related species indicate that their ancestors could have arisen from more closely related lineages.

The maternal to zygotic transition (MZT) is a separate developmental period that begins with the elimination of maternal transcripts, continues through the production of zygotic transcripts, and concludes with the first major morphological requirement for zygotic transcripts in embryo development.The α-Amanitin as transcript inhibition factor was used to determine the zygotic genome switch in sterlet embryos. The transition from maternal to zygotic type is characterized by the elimination of maternal transcripts and continues through the production of zygotic transcripts. Thus, the embryo has grown enough to become an autonomous system independent of maternal factors. In sterlet it was observed after the tenth cleavage during late blastula, when blastomeres in the animal pole are surpassed 1000 cells.

Mid-blastula transition (MBT) in early embryogenesis can be defined as a time point characterized by cell cycle lengthening, loss of synchrony and acquisition of cell motility Morphological changes during mid-blastula transition represent milestones in the development of metazoans, since each cell transmits a perfect copy of its genome at each division. We opted to use oocytes of crosses sterlet *A. ruthenus* and Russian sturgeon *Acipenser gueldenstaedtii*, since the hybridization results in increased DNA content in their hybrid offspring compared to parental species *A. ruthenus* making the embryo a useful model for investigation of changes in the timing of early development. Nucleous vizualization by 4'-6-diaminido-2-phenylindole (DAPI) staining showed that cells divided synchronously at a constant rate until MBT at the ninth cell cycle in control sterlet embryos that corresponds to 1000 cell stage (13 hpf). The sterlet x Russian sturgeon hybrid embryos showed transition from synchronous to asynchronous division at the eighth cell cycle which is the 512 cells stage (12 hpf). In both sterlet and hybrid embryos, the transition occurred within 1 h. Thus, our study confirmed hypothesis the MBT in sturgeon is governed by the ratio of nucleus to cytoplasm, which can be controlled using hybridization, induction of polyspermy or injecting plasmid DNA.

Embryos of sturgeon injected with α-Amanitin also showed cell cycle kinetics similar to controls, with no delay or malformation during cleavage, which most likely indicates that MBT in the sturgeon proceeds independently of onset of zygotic transcripts production.

The results and observations presented in this study demonstrate the path from an egg to a developed embryo, which are the basis for improving the production methods and preservation of sturgeons listed in the IUCN Red List, and which is equally important, provide the fundamental knowledge about the nature of sturgeons.

Czech summary

Základ mateřských faktorů u jesetera: od vajíčka po embryo

Kseniia Pocherniaieva

Pokles početnosti divokých populací jesetera v posledních desetiletích vedl k rostoucímu zájmu o využití různých biotechnologických postupů vedoucích k ochraně nebo obnově těchto ohrožených druhů. Produkce chimér, transplantace zárodečných buněk nebo produkce pomocí tzv. "náhradního rodiče" by mohly rozšířit spektrum technik používaných k záchraně druhu či vést k obnově rybích populací. Úspěšná aplikace těchto metod u jeseterů vyžaduje znalost jeho embryonálního vývoje a informace o struktuře a charakteristikách samotného jeseteřího oocytu. K odhalení intracelulární geometrie, morfogenetických aspektů, mechanizmů organizace mateřských determinantů a jejich pozdější reorganizace byly v rámci této práce použity techniky qPCR tomografie, inhibice transkripce a vizualizace jader.

qPCR tomografie se ukázala jako spolehlivá technika pro stanovení úlohy genů detekovaných v animální a vegetativní hemisféře oocytů jesetera a také pro identifikaci profilů těchto genů u ranných vývojových stadií embryí jesetera. qPCR tomografie byla v zásadě provedena ve dvou krocích: předběžné kroky tomografie a samotná RT-PCR. Optimálně orientované oocyty byly rozřezány na 30 µm a sekce (prokrývající jak animální, tak vegetativní pól), u který byla následně provedena celková extrakce RNA. 12 vybraných maternálních genů: [beta-actin (*actb*), peptidylprolyl isomerase A (*ppia*), 5'-aminolevulinate synthase (*alas1*), succinate dehydrogenase complex flavoprotein subunit A (*sdha*), tyrosine 3-monooxygenase (*ywhae*), DND microRNA-mediated repression inhibitor 1 (*dnd*), DEAD-box helicase 4 (*vasa*), vegt protein (*vegt*), wingless-type MMTV integration site family member 11 (*wnt11*), DEAD-box helicase 25 (*ddx25*), orthodenticle homeobox 1 (*otx1*) a growth differentiation factor 1 (*gdf1*)] bylo sledováno pomocí RT-PCR.

Identifikovány byly dvě skupiny transkriptů klasifikovaných jako animální nebo vegetativní geny s jasným gradientem. Primární geny zárodečné plasmy – markery jako *dnd*, *vasa*, *ddx25* byly lokalizovány směrem k vegetativnímu pólu. To vysvětluje, kde presně se nachází prapohlavní zárodečné buňky v oocytech jesetera malého. Dalším aspektem použití této techniky byla srovnávací analýza profilů RNA v oocytech drápatky *Xenopus laevis* a jesetera malého *Acipenser ruthenus*. Byla zjištěna jednoznačná podobnost v lokalizaci molekul mRNA u těchto dvou druhů. Takovéto podobnosti v expresních profilech vzdáleně příbuzných druhů naznačují, že jejich dávní předkové mohli vzniknout z více příbuzných linií.

α-Amanitin jako transkripční inhibiční faktor byl použit ke stanovení zygotického genomového přechodu v embryích jesetera malého. Přechod z mateřského na zygotický typ transkripce je charakterizován eliminací mateřských transkriptů a pokračuje produkcí zygotických transkriptů. U jesetera malého byl tento přechod identifikován po desátém štěpení – během pozdní blastuly, kdy počet buněk v blastomeře překročil 1 000.

Morfologické změny během přechodu mid-blastuly představují milníky ve vývoji metazoanů, protože každá buňka přenáší perfektní kopii svého genomu v každé divizi. Embrya vzniklá křížením jiker jesetera malého *A. ruthenus* a spermatu jesetera ruského *Acipenser gueldenstaedtii* druhů s odlišnou ploidní úrovní byla vytvořena za účelem zvýšení obsahu DNA v jádrech buněk. Nukleární vizualizace barvením 4'-6-diaminido-2-fenylindolem (DAPI) ukázala, že se buňky dělí synchronně konstantní rychlostí až do MBT v devátém buněčném cyklu u kontrolních embryí, která odpovídají 1 000 buněčnému stadiu (13 hpf). Hybridní embrya jesetera malého a ruského vykazovala přechod od synchronního k asynchronnímu dělení v osmém buněčném cyklu, což je stadium 512 buněk (12 hpf). U jesetera malého i hybridních embryí došlo k přechodu během 1 hodiny.

Embrya jesetera injikovaného α-Amanitinem také vykazovala kinetiku buněčného cyklu podobnou kontrolám, a to bez zpoždění nebo malformace během štěpení, což s největší pravděpodobností naznačuje, že MBT u jesetera probíhá nezávisle na začátku produkce zygotických transkriptů.

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List of publications

Peer-reviewed journals with IF

Naraine, R., Abaffy, P., Sidova, M., Tomankova, S., **Pocherniaieva, K.**, Smolik, O., Kubista, M., Psenicka, M., Sindelka, R., 2020. NormQ: RNASeq normalization based on RT-qPCR derived size factors. Computational and Structural Biotechnology Journal 18, 1173–1181. (IF 2019 = 6.018)

Pocherniaieva, K., Guralp, H., Saito, T., Pšenička, M., Tichopád, T., Janko, K., Kašpar, V., 2019. The timing and characterization of maternal to zygote transition and mid-blastula transition in sterlet *Acipenser ruthenus* and *A. ruthenus* x *Acipenser gueldenstaedtii* hybrid. Turkish Journal of Fisheries and Aquatic Sciences 19, 77–84. (IF 2018 = 0.738)

Pocherniaieva, K., **Š**idova, M., Pšenička, M., Saito, T., Šindelka, R., Kašpar, V., 2018. Comparison of oocyte mRNA localization patterns in sterlet *Acipenser ruthenus* and African clawed frog *Xenopus laevis.* Journal of Experimental Zoology part B Molecular and Developmental Evolution 330, 181–187. (IF 2017 = 2.432)

Guralp, H., **Pocherniaieva, K.**, Blecha M., Policar, T., Pšenička, M., Saito, T., 2017. Development, and effect of water temperature on development rate, of pikeperch *Sander lucioperca* embryos. Theriogenology, 104, 94–104. (IF 2016 = 1.986)

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Abstracts and conference proceedings

Fatira, E.,Labbe, C., Depince, A., Iegorova, V., **Pocherniaieva, K**., Güralp, H., Havelka, M., Psenicka, M., Saito, T., 2017. Single and multiple somatic cells nuclear transfer in critically endangered species, sturgeon. 6th International Workshop on the Biology of Fish Gametes. September 4–7, 2017, Vodnany, Czech Republic. (oral presentation)

Pocherniaieva, K., Guralp, H., Pšenička, M., Saito, T., Kašpar, V., 2016. The mid-blastula transition in sturgeon. FABA 2016: International Symposium on Fisheries and Aquatic Sciences. November 3–5, 2016, Antalya, Turkey. (oral presentation)

Pšenička, M., Guralp, H., **Pocherniaieva, K.**, Linhartova, Z., Saito, T., 2016. Generation of germline chimera in sturgeon. FABA 2016: International Symposium on Fisheries and Aquatic Sciences. November 3–5, 2016, Antalya, Turkey. (poster presentation)

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Pocherniaieva, K., Šidova, M., Pšenička, M., Saito, T., Šindelka, R., Kašpar, V., 2015. qPCR tomography of the *Acipenser ruthenus* oocyte. 5th International Workshop on the Biology of Fish Gametes. September 7–11, 2015, Ancona, Italy. (oral presentation)

Guralp, H., **Pocherniaieva, K.**, Blecha, M., Psenicka, M., Saito, T., 2013. Embryonic stages and primordial germ cells 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)

Guralp, 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

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

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

International conferences	**Year**
Pocherniaieva, K., Šidova, M., Pšenička, M., Saito, T., Šindelka, R., Kašpar, V., 2015. qPCR tomography of the *Acipenser ruthenus* oocyte. 5th International Workshop on the Biology of Fish Gametes, 7–11 September 2015, Ancona, Italy. (oral presentation)	2015
Pocherniaieva, K., Güralp, H., Pšenička, M., Saito, T., Kašpar, V., 2016. The mid-blastula transition in sturgeon. FABA 2016: International Symposium on Fisheries and Aquatic Sciences, 3–5 November, 2016, Antalya, Turkey. (oral presentation)	2016

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Prof. Katsutoshi Arai – *In situ* hybridization. Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Science, Hokkaido University, Hakodate, 041-8611, Japan (2 weeks)	2014
Dr. Catherine Labbé – Germ-line cells transcriptome evaluation of the nuclear-cytoplasmic hybrids, Fish Physiology and Genomics Institute, INRA LPGP, Campus de Beaulieu F-35042 Rennes, France (6 weeks)	2015
Prof. Finn-Arne Weltzien – Single-cell qPCR analysis of medaka pituitary cells. Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, NMBU, Ullevålsveien 72 0454 Oslo, Norway (2 months)	2017

Pedagogical activities	**Year**
Training of students in laboratory of molecular biology at USB FFPW in range of 70 teaching hours	2015–2016

Summer school students supervision	**Year**
Anastasiia Kharandiuk, Species specific primers for identification of hybrids, clones, chimeras	2015
Sakura Tanaka, Nuclei visualization with DAPI in sturgeon	2015

Foreign courses, trainings and seminars	Year
AQUAGAMETE training course, Techniques for fish germline cryobanking, Institute of Marine Science of Andalusia, Cadiz, Spain	2013
AQUAGAMETE training course, Molecular basis of fish gamete quality, INRA LPGP, Rennes, France	2015

Curriculum vitae

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PROFESSIONAL EXPERIENCE

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2011–2012 Laboratory technician, Chair of Biological and Medical Physics, V.N. Karazin Kharkov National University, Kharkiv, Ukraine
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September, 2014 Prof. Katsutoshi Arai – *In situ* hybridization. Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Science, Hokkaido University, Hakodate, 041-8611, Japan

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March–May, 2017 Prof. Finn-Arne Weltzien – Single-cell qPCR analysis of medaka pituitary cells. Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, NMBU, Ullevålsveien 72 0454 Oslo, Norway

COMPLETED COURSES

2013 Real-Time PCR Course, Training skills course, Brno, Czech Republic

2013 AQUAGAMETE training course, Techniques for fish germline cryobanking, Institute of Marine Science of Andalusia, Cadiz, Spain

2014 Course on Biological Specimen Preparation for Electron Microscopy, Training course specify on SEM and TEM, České Budějovice, Czech Republic

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2016 Single-cell analysis and Hands-on qPCR course, TATAA Biocenter, Prague, Czech Republic