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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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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

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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.

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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) (Fig. 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 (Fig. 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 (Fig. 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 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).

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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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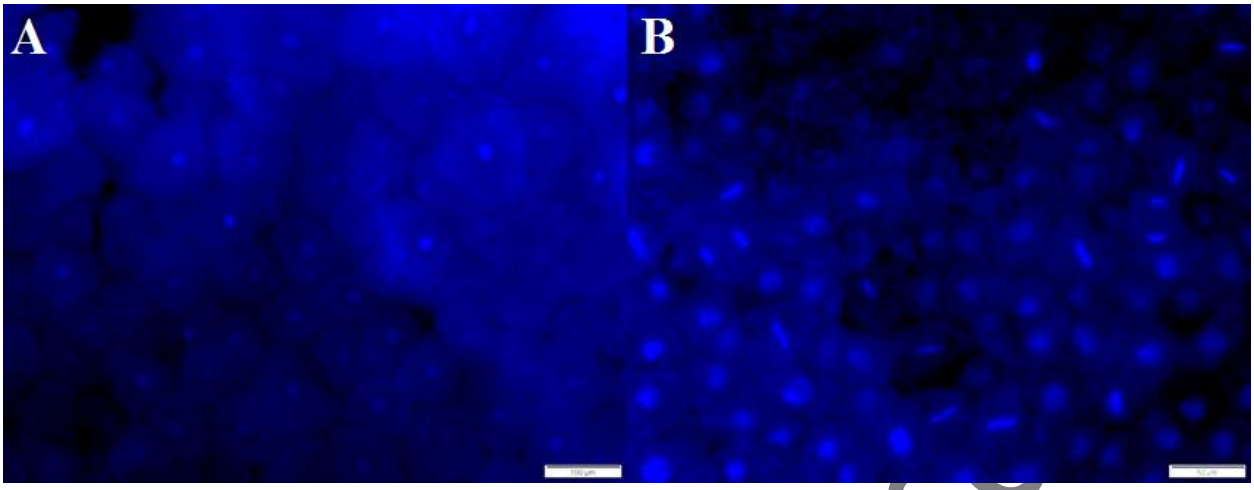

Figure 1. Fluorescence micrographs of cleavage cycle in *Acipenser ruthenus* embryos. (A) Synchronous S phase in majority of nucleus in the 9th cell cycle (13 hpf), scale bar = 100 µm. (B) Asynchronous mitotic phases represented by three different phases (pro-, meta- and telophase) of nuclei in blastomeres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

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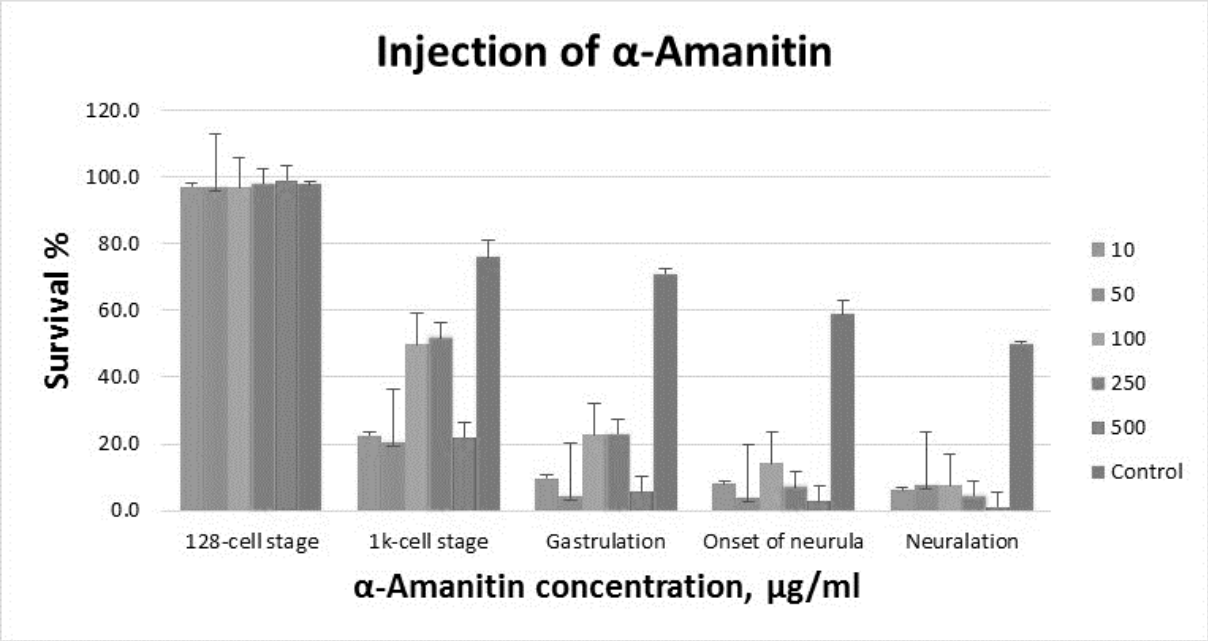

Figure 2. Survival of *Acipenser ruthenus* embryos during development after injection with *α*-Amanitin. Error bars indicate mean ± standard deviation of results from serial experiments.