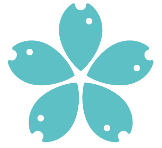

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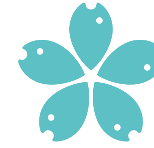
Nuclear transplantation in sturgeon eggs

Effrosyni Fatira



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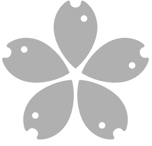

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CHAPTER 1

GENERAL INTRODUCTION

Introduction

The nuclear transfer is an animal cloning technique and involves two steps; removing the nucleus from a recipient oocyte (unfertilized egg) and injecting a nucleus comes from a different individual, whi ch contains the genome to be cloned. The newly constructed cell will divide normally, replicating the new DNA, creating an individual from the donor origin only (Briggs and King, 1952; Tung et al., 1963; Mc Grath and Solter, 1983). Depending on the nucleus cell type injected, the nuclear transfer is categorized as embryonic or somatic. When the donor nucleus is coming from an embryonic cell, the nuclear transfer is called Embryonic Cell Nuclear Transfer (ECNT), while when it is coming from an adult somatic cell, it is called Somatic Cell Nuclear Transfer (SCNT) (Labbé et al., 2013).

The principle of the SCNT technique is that the differentiated donor cell can be restored to full totipotency when it is transplanted in the prosperous environment of a recipient egg (Zhu and Sun, 2000). This technology allows the restoration of valuable genetic resources from somatic material when both sperm and oocytes, or embryos, are not available. Early development of the reconstructed embryos relies on the cytoplasmic components of the recipient enucleated oocyte (mitochondria, maternal RNA, proteins) and the embryos are named as nucleocytoplasmic hybrids (St John et al., 2004; Labbé et al., 2013; Chênais et al., 2014). The importance of the SCNT technique is that genes in the nucleus of a mature differentiated somatic cell are still able to revert to an embryonic totipotent state (Evans and Kaufman, 1981; Wilmut et al., 1997). To date along with the induced pluripotent cells are powerful techniques that can endow the somatic cell genome with totipotency (Takahashi and Yamanaka 2006).

Depending on the species' origin of the donor somatic nucleus and the egg recipient, the SCNT is characterized as intraspecific (when both come from the same species), or interspecific (when they come from different species). From the perspective of biology, the first offers a powerful approach to study the pluripotency or totipotency of the differentiated nuclei. On the other hand, taking advantage of the developmental differences between two species, interspecific SCNT (iSCNT) provides a probe to study the interaction between the nucleus and cytoplasm involved in development (Sun et al., 2005). From the perspective of aquaculture, the utility of intraspecific cloning can have great advantages for replication of an elite fish. In sturgeon case that favorable fish could be a female individual with big ovary capacity that can produce big amount of roe that subsequently will be processed into caviar. In the case of interspecific cloning, producing cloned endangered sturgeon species that are favorable for caviar consumption (Hochleithner and Gessner, 2019), like is the case of Russian sturgeon and beluga (IUCN, 2019), utilizing as a recipient a smaller sturgeon like the sterlet that categorized as vulnerable (IUCN, 2019), is very beneficial economically.

In the present Ph.D. study, only the sturgeon SCNT technique applied, while the ECNT was omitted for two reasons. This happened because practically it is not easy to obtain an embryonic cell from blastula sturgeon embryos because they are exhibiting a holoblastic cleavage pattern (embryonic cleavage appears from animal to vegetal pole) (Dettlaff et al., 1993). In addition, from our experience, the egg's internal pressure is too high and a "rough" manipulation, such is the isolation of a blastomere, will cause the complete damage of the embryo. Moreover, after *in vitro* fertilization of the donor embryos it takes approximately 3–4 hours for the first cleavage, a significant delay that causes a decrease in the recipient egg's quality, one major factor in cloning efficiency (Cibelli et al., 2001). The second reason not to apply the ECNT technique is ethical. The ECNT demands the sacrifice of many sturgeon embryos in order to collect donor nuclei for the transplantation procedure, and this is contradictory to the fact that sturgeons are categorized as endangered species (IUCN, 2019). For that reason,

the present study performed SCNT using differentiated cells coming from a fin tissue, because fin sampling does not cause any irreparable damage to the fish (Akimenko et al., 2003).

History of the animal cloning technique

The nuclear transfer saga goes back to as far as the fifties, with the pioneering work of Briggs and King (1952) in which the nucleus of frogs, *Rana pipiens* embryonic cells were injected into enucleated unfertilized eggs, resulting in the normal development of embryos. This work demonstrated that blastula cell nuclei could be directed to the development of complete tadpoles when transferred into the cytoplasm and activated enucleated frog egg. It was mainly devoted to the understanding of the totipotency and the differentiation of the embryonic cell nucleus during embryo development.

In contrast to ECNT, the SCNT technique has not been succeeded for long because of the inability of the differentiated cell nucleus to be reprogramed in order to sustain embryo development after the transplantation. The first conclusive success was obtained in mammals with the birth of a female domestic sheep called Dolly (Wilmut et al., 1997). This report was the first demonstration that an adult cell still retains all the information necessary to generate an entire organism. In this research, Wilmut et al. (1997) used a nucleus from cultured mammary gland cells. This usage opened new possible applications for nuclear transfer technology, including the perspective to use cryobanked somatic cultured cells to reconstruct endangered animals (Loi et al., 2007). Today, SCNT is almost routinely applied in mammals to produce fertile adults, especially in sheep, cattle and horses, in contrast to fish (Lee et al., 2002; Liu et al., 2002; Ju et al., 2003; Bubenshchikova et al., 2007, 2008; Kaftanovskaya et al., 2007; Wakamatsu, 2008; Siripattarapravat et al., 2009; Le Bail et al., 2010).

Cloning technology in fish has been developed with embryonic cells since the 1960s (Tung et al., 1963; 1965; Gasaryan et al., 1979), and viable offspring have been produced (Shaoyi et al., 1991). In fish species, however, the success of SCNT has been limited in model species and the success rate is very low. Since the first adult cloned zebrafish, *Danio rerio* obtained by Lee et al. (2002) from long-term cultured fibroblast cells, the success of 2% has not been increased using somatic cells. In general, fish SCNT is mostly unsuccessful in producing living adult clones (Ju et al., 2003; Kaftanovskaya et al., 2007; Wakamatsu, 2008; Siripattarapravat et al., 2009). There are a lot of constraints that cause a low success rate of producing clones.

Nuclear transplantation in fish was first reported in loach, *Misgurnus fossilis* by Gasaryan et al. (1979). In their study, ECNT into non-enucleated or enucleated eggs was conducted, and the nuclear transplants grew up to the hatchling stage. Extensive studies on ECNT, mainly in cyprinid fish have been performed, and nucleocytoplasmic hybrids by transplanting the embryonic nuclei of one species into enucleated eggs of another species have been produced (Yan, 1998; Sun et al., 2005; Pei et al., 2007). Another case was the transplantation of nucleus from embryonic cells into unfertilized non-enucleated eggs obtaining cytoplasmic hybrids which were triploid and infertile. In the triploid cytoplasmic hybrids, endogenous and exogenous genetic markers originating from donor nuclei were expressed normally (Niwa et al., 1999). Diploid and fertile adult fish were obtained from the nuclear transfer of blastula nuclei to enucleated (Wakamatsu et al., 2001) and non-enucleated (Bubenshchikova et al., 2005) unfertilized eggs in medaka, *Oryzias latipes*. Additionally, adult zebrafish were cloned from cultured embryonic cell nuclei by Lee et al. (2002). Since then, several groups have reported various successes with cultured somatic cells in fish (Kaftanovskaya et al., 2007; Bubenshchikova et al., 2008; Wakamatsu, 2008; Siripattarapravat et al., 2009; Le Bail et al., 2010; Perez-Camps et al., 2010; Siripattarapravat et al., 2010; Luo et al., 2011; Tanaka et al., 2012), but most of the developments barely went beyond hatching.

In 1984, the first somatic cell cloned fish was derived from short-term cultured kidney cells of triploid carp, *Cyprinus carpio* (Chen et al., 2010). In this experiment, two rounds of nuclear transfer were carried out. In the first round, the nucleus (3n) was transferred into the enucleated eggs (2n) of crucian carp, *Carassius carassius* resulting in 41% of the transplanted eggs to develop into blastulae without further development. Nuclei from the blastulae were taken for the second round of transfer into another enucleated egg recipients (2n) resulting in the production of 8 gastrula stage transplants. Among them, one developed into a fertile female fish (1.2%) with normal morphological features of crucian carp and showed triploidy. The result suggested that some nuclei of somatic cells, following two rounds of nuclear transplantation, could be reprogrammed to totipotent status as the zygotic nucleus does (Yan and Sun, 2000). Successes on SCNT conclusively revealed that differentiated adult cells still remain totipotent and maintain the whole genome to support normal development to term. Promising results have been obtained more recently with somatic cells in zebrafish (Lee et al., 2002; Siripattarapravat et al., 2009), medaka (Bubenshchikova et al., 2007; Kaftanovskaya et al., 2007) and goldfish, *Carassius auratus* (Le Bail et al., 2010; Tanaka et al., 2012), whereas with low developmental success of the clones. Therefore, although promising, the technology of the somatic cell nuclear transfer in fish deserves further improvement and understanding of the bottlenecks, both at the technical and at the biological level (Chênais et al., 2014).

Benefits and technical problems of the technique

The nuclear transfer has potential applications in animal science and regeneration medicine for humans and also allows the simple propagation of "elite" strains of animals (Meissner and Jaenisch, 2006). For instance, in the case of sturgeon species, there are some favorable strains with a big ovarian cavity that can produce a bigger amount of eggs, as well as sturgeon species most commonly exploited for caviar, the Russian sturgeon, *Acipenser gueldenstaedtii* and beluga, *Huso huso* (Hochleithner and Gessner, 2012).

As somatic cells can be proliferated and gene-modified *in vitro*, SCNT has been expected to contribute extensively to the farm animal production industry, drug production, regenerative medicine and conservation of invaluable genetic resources (Vajta, 2007; Oback, 2008). Besides its broad practical applications, SCNT can provide unique and interesting experimental systems for genomic research, especially in epigenetics, to learn how the somatic cell genome is reprogrammed into a state equivalent to that of the fertilized oocyte: the so-called totipotent state (Gurdon and Wilmut, 2011).

Interspecific nuclear transfer has been used to study developmental plasticity and nuclear reprogramming of the donor nucleus and to generate reprogrammed stem cells from differentiated cells (Gurdon and Wilmut, 2011). Furthermore, the possibility to preserve endangered species by interspecific nuclear transfer using domestic animals as oocyte recipients has been reported, indicating another potentially useful application of nuclear transfer (Lanza et al., 2000). In the case of critically endangered sturgeon species, sometimes it is very difficult to wait for the sexual maturation of the animal to collect eggs or sperm. For example, the Russian sturgeon and beluga that are both classified as critically endangered species (IUCN, 2019), they start reproducing late, at 10–16 and 8–13 years (Gesner et al., 2010a) for females and males respectively, and at 15–18 and 10–15 years for beluga, respectively (Gesner et al., 2010b). The sterlet, *A. ruthenus* is classified as a vulnerable species (IUCN, 2019) and displays characteristics that make it a model species of the sturgeon family (Saito et al., 2014). Sterlet has early reproduction activity (5–8 years in females and at 3–5 years in males) (Gesner et al., 2010c) and the female individuals spawn every 1–2 years (Gesner et al., 2010c) while Russian sturgeon and beluga spawn at 4–6 (Gesner et al., 2010a) and 3–4 years (Gesner et al., 2010c) intervals, respectively.

Interspecific cloning using somatic cells has been reported in vulnerable or threatened mammal species like gaur calf, *Bos gaurus* (Lanza et al., 2000; Srirattana et al., 2012), European mouflon, *Ovis orientalis* (Loi et al., 2001), black-footed cat, *Felis nigripes* (Gómez et al., 2006) and more recently in Bactrian camel, *Camelus bactrianus* (Wani et al., 2017). However, as far as we know, this technique has not been applied to an endangered species until the present Ph.D. study. Sturgeons are known as a relict group of species and are amongst the most endangered organisms due to habitat degradation, over-harvesting and illegal fishing for their roe. Therefore, it is considered one of the most suitable candidates for cloning in practice (Fatira et al., 2018; Fatira et al., 2019). Despite the demonstrated advantages of the method, SCNT is a challenging multi-step technique with low success even in model fish species (Lee et al., 2002; Ju et al., 2003; Kaftanovskaya et al., 2007; Wakamatsu, 2008; Siripattarapravat et al., 2009; Luo et al., 2011).

In the course of cloning study in model fish species, it has been proposed that there are some key steps to be solved that are necessary for the success of the nuclear transfer. The steps summarizing in three procedures: 1) the isolation of single somatic cells prior to transplantation, 2) the preparation of the recipient oocytes, and 3) the establishment of "microinjection technique".

The fin can be harvested even before the sexual maturation of the individual (Chênais et al., 2014) and this is very important for sturgeon species, as already have been mentioned their late gonadal maturation. In addition, the fin is the easiest tissue to sample and the least damaging to the fish (Akimenko et al., 2003; Labbé et al., 2013). Interspecific SCNT application to endangered fish species has a great advantage, as the reconstruction of the critically threatened species can be achieved after a single fin-cell is transplanted in the egg-cytoplasmic environment of species whose eggs are easily available in farms. If the iSCNT technology realized, reconstruction of animals can be possible by transplantation of a fin cell from a critically endangered sturgeon like a Russian sturgeon or beluga into model species egg recipient like sterlet (Fatira et al., 2018; Fatira et al., 2019).

Regarding the step of oocytes' preparation, whatever its nuclear DNA background, the developing embryo will use the cytoplasmic components of the recipient oocyte (mitochondria, its small DNA, proteins, maternal mRNAs) and therefore the reconstructed embryos are referred to as nucleocytoplasmic hybrids (Moritz and Labbé, 2008; Labbé et al., 2013). Before the injection, oocyte must be captured in metaphase II and incubation conditions must maintain its quality intact during the whole microinjection process. Accidental activation will trigger maturation/mitosis promoting factor degradation and the subsequent environment in which the somatic nucleus is exposed will be less favorable to reprogramming (Le Bail et al., 2010). With respect to the last step, in fish eggs, donor cells must be transplanted at the animal pole region of activated or non-activated eggs. According to Bail et al. (2010), transplantation via micropyle without egg activation seems to be the best for producing clone fish so far. However, in this case, micro-needle must be customized for the penetration of an egg chorion with special care to remaining very close to the egg surface. Any other location will impair development.

It was believed that the recipient oocyte should be enucleated before nuclear transplantation in order to ensure the developing embryo will only bear the genome of the donor nucleus. Interestingly, several authors by-passed the enucleation step during the fish nuclear transfer in goldfish, zebrafish, weatherfish, *Misgurnus anguillicaudatus*, medaka, and the resulted developing cytoplasmic embryos carried only the genome of the donor (Ju et al., 2003; Bubenshchikova et al., 2005; Bubenshchikova et al., 2007; Tanaka et al., 2012; Kaftanovskaya et al., 2007; Le Bail et al., 2010). These results provide hope in fish cloning, because the oocyte structure makes enucleation very difficult: oocytes are large and opaque, they contain

bulky nutritional reserves (the yolk), a dense cytoplasm (the ooplasm), and a thick protective envelope around the oocyte (the chorion) (Iwamatsu, 2004; Lubzens et al., 2010). These characteristics prevent the visualization of the maternal genome by transparency and its aspiration for enucleation.

In fish, most groups use blind enucleation after egg activation (Yan et al., 1991; Lee et al., 2002; Sun et al., 2005; Luo et al., 2011), once meiosis resumption has triggered the release of the second polar body. Normally, the second polar body extrudes only after the fertilization takes place. Taking the second polar body as a landmark of the forming female pronucleus underneath, the researchers with the use of a glass microcapillary aspirate the cytoplasm under the polar body according to the original method of Briggs and King (1952). However, these oocytes are less suitable for donor DNA reprogramming because the aspiration of the female pronucleus is associated with loss of essential developmental factors such as maternal mRNAs, mitochondria, and proteins (Chênais et al., 2014). Overall, in addition to time-consuming, fish enucleation is a very problematic issue for the success of nuclear transfer and embryonic development of the clone. Irradiation of the recipient egg by UV, X or gamma rays is another method used to inactivate the maternal genome. Several groups used the procedure for enucleation prior to nuclear transfer (Gasaryan et al., 1979; Hongtuo and Chingjiang, 2001; Wakamatsu et al., 2001; Liu et al., 2002). More recently, Siripattarapravat et al. (2009, 2010) proposed a refined irradiation method where the animal pole of an inactivated egg was submitted to focused laser irradiation whose narrowness aimed to reduce the damages inflicted by irradiation to the non-genomic molecules of the eggs. Therefore, this method succeeded in inactivating the maternal metaphase at a more appropriate recipient stage, but the adaptation of this method to species other than zebrafish was never reported, likely because of the difficulty tuning of the laser on different egg types (Rouillon et al., 2019).

In fish species, the success of SCNT has been limited in model species, such as zebrafish, medaka, carp, and goldfish, due to the accessibility of the eggs and the ease of its manipulation. However, even in these model species, there are still many biological and technical constraints, i.e. mechanical damage of donor nuclei and asynchrony between the cell cycles of the recipient egg and donor nucleus (Sun et al., 1992; Campbell et al., 1996; Cibelli et al., 1998; Kato et al., 1998; Meissner and Jaenish, 2006; Wakamatsu, 2008) and quality of recipient eggs (Chen et al., 2010). Nuclear transfer primarily addresses a biological rather than a methodological level. Indeed, most researchers suspect that the DNA reprogramming process taking place on gametic chromatin during normal embryo development (Mhanni and Mc Gowan, 2004) does not operate properly when the chromatin is from somatic origin (Pei et al., 2007, 2008, 2009; Liu et al., 2008; Le Bail et al., 2010; Siripattarapravat et al., 2010). How the exogenous chromatin can be modified in order to undergo a proper reprogramming upon development initiation is currently the main focus for research in the field (Pei et al., 2007, 2008, 2009; Liu et al., 2008; Luo et al., 2009, 2011).

A key factor in cloning-associated abnormalities probably involves inadequate epigenetic reprogramming of the donor genome. DNA methylation, one of the best-studied epigenetic modifications, is known to be aberrant in many clones (Meissner and Jaenish, 2006). DNA methylation provides heritable information to the DNA that is not encoded in the nucleotide sequence and participates in a diverse range of cellular functions and pathologies, including tissue-specific gene expression, cell differentiation, genomic imprinting, X-chromosome inactivation, regulation of chromatin structure, carcinogenesis and aging (Bird, 2002). Apart from DNA methylation, histone acetylation, chromatin remodelling and the regulation of non-coding RNA, such as microRNA (miRNA) and long non-coding RNA (lncRNA) are other epigenetic modifications. Recent studies have shown that lncRNAs play an important role in epigenetic modification and pluripotency maintenance in somatic cell reprogramming of

human pluripotent stem cells (Flynn and Chang, 2014; Durruthy-Durruthy et al., 2016) by blocking the degradation of miRNA to OCT4, SOX2 and NANOG (Loewer et al., 2010). Nanog, Sox2, Oct4, Klf4 and c-Myc, there are known as pluripotency-associated genes and they are silenced in differentiated somatic cells but expressed in early embryonic undifferentiated stem cells (Guenther, 2011). For this reason, these genes should be reactivated for the successful somatic cell reprograming. Recently, Hu et al. (2018) provided the first evidence of fam60al as a novel factor involved in somatic cell nuclear reprogramming in zebrafish. The gene has been found to initiate the reprogramming process instead of maintaining pluripotency in zebrafish.

The SCNT technique forces the somatic cell genome to be reprogrammed directly to a totipotent state by bypassing these erasing steps, and this might make the technique prone to epigenetic errors and cause frequent death and loss of embryos (Ogura et al., 2013). Complete reprogramming of somatic cell depends on whether epigenetic modification of donor cells can be restored to the state of totipotent stem cells following their transfer into recipient enucleated eggs in SCNT embryos (Rideout et al., 2001; Beaujean et al., 2004). Most groups report some successful developments up to the mid-blastula (MBT) stage of the reconstructed embryos (Wakamatsu, 2008; Le Bail et al., 2010; Siripattarapravat et al., 2010; Luo et al., 2011) and that dramatic losses are ensuing at later stages. Developmental failures after MBT indicate that the reprogramming of the somatic chromatin into an embryonic pattern was not optimal. This explanation is further illustrated by the work of Liu et al. (2008), Pei et al. (2008), and Luo et al. (2009). These authors observed differential expression of numerous genes between embryos obtained after fertilization and after nuclear transfer.

In the context of genome resource preservation, the main issue with any reconstruction technology is to ensure that the genome of the valuable individual is correctly represented in the reconstructed offspring. It is therefore important to carefully characterize the genetic background of the so-called clones after nuclear transfer. Many groups used donor fish that are playing phenotypic traits easy to analyze, such as skin pigmentation (Niwa et al., 1999; Siripattarapravat et al., 2009) or transgene expression (Zhu and Sun, 2000; Lee et al., 2002). The Acipenseridae family contains species with an interesting specificity; i.e. different ploidy between them. For instance, sterlet, beluga, and the European sea sturgeon, A. sturio are diploid (Birstein and Vasil'ev, 1987; Fontana et al., 1998; Tagliavini et al., 1999) while the Sakhalin, A. mikadoi and Russian sturgeon are tetraploid (Fontana et al., 1996; Vasil'ev et al., 2009), and the shortnose sturgeon, *A. brevirostrum* is hexaploid (Fontana et al., 2008). Therefore, after sturgeon iSCNT between species with different ploidy levels, it would be wised to analyze the resulting nuclear transplants (NT) in their ploidy level.

Objectives of the thesis

The acipenseriformes, including sturgeons, is the oldest order within the Actinopterygii. This order is frequently referred to as ''living fossils'' in the literature. The fossil record of sturgeon dates back to the Upper Cretaceous (Grand and Bemis, 1991), and mitochondrial DNA analysis suggested that they had diverged from an ancient, pre-Jurassic teleost lineage approximately 300 million years ago (Inoue et al., 2005). The Acipenseridae is an ancient family that faces internal and external threats including the loss of species genetic integrity through frequent interspecific hybridization (Ludwig et al., 2009), habitat degradation, and overfishing for their roe processed into caviar (Birstein et al., 1997). A dramatic decrease in sturgeon populations attracted the attention of the International Union for Conservation of Nature (IUCN) that categorized them as the most critically endangered, more than any other group of species. Indeed, all 27 sturgeon species are on the IUCN Red List of threatening species with 17 categorized as critically endangered and four considered to be extinct (IUCN,

2019). Therefore, establishment of the SCNT technique that can aid conservation of species on the verge of extinction (Wildt, 1992; Solti et al., 2000; Li et al., 2006; Oh et al., 2008; Gómez et al., 2009; Labbé et al., 2013; Fatira et al., 2018; Fatira et al., 2019) is a necessity.

The current Ph.D. study is devoted to the introduction and optimization of the sturgeon cloning technique pursuing the following objectives:

1. To apply the iSCNT on sturgeon species, establishing the crucial first steps by adjusting the cloning-methodology in sturgeon's biology.
2. To improve the sturgeon iSCNT in order to make it a feasible tool for regeneration of sturgeon used for meat and caviar consumption in aquaculture.

References

Akimenko, M.A., Mari-Beffa, M., Becerra, J., Geraudie, J., 2003. Old questions, new tools, and some answers to the mystery of fin regeneration. Dev. Dynam. 226, 190–201.

Beaujean, N., Taylor, J., Gardner, J., 2004. Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer. Biol Reprod. 71, 185–93.

Bird, A.P., 2002. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res. 8, 1499–1504.

Birstein, V.J., Vasiľev, V.P., 1987. Tetraploid-octoploid relationships and karyological evolution in the order *Acipenseriformes* (Pisces): karyotypes, nucleoli, and nucleolus-organizer regions in four acipenserid species. Genetica 73, 3–12.

Birstein, V.J., Bemis, W.E., Waldman, J.R., 1997. The threatened status of acipenseriform species: a summary. Environ. Biol. Fishes 48, 427–435.

Briggs, R., King, T., 1952. Transplantation f living nuclei from blastula cells into enucleated frog's eggs. Zoology 38, 455–463.

Bubenshchikova, E., Ju, B., Pristyazhnyuk, I., Niwa, K., Kaftanovskaya, E., Kinoshita, M., Ozato, K., Wakamatsu, Y., 2005. Generation of fertile and diploid fish, medaka (*Oryzias latipes*), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. Cloning Stem Cells 7, 255–264.

Bubenshchikova, E., Kaftanovskaya, E., Motosugi, N., Fujimoto, T., Arai, K., Kinoshita, M., Hashimoto, H., Ozato K., Wakamatsu Y., 2007. Diploidized eggs reprogram adult somatic cell nuclei to pluripotency in nuclear transfer in medaka fish (*Oryzias latipes*). Dev. Growth Differ. 49, 699–709.

Bubenshchikova, E., Kaftanovskaya, E., Hattori, M., Kinoshita, M., Adachi, T., Hashimoto, H., Ozato, K., Wakamatsu, Y., 2008. Nuclear transplants from adult somatic cells generated by a novel method using diploidized eggs as recipients in medaka fish (*Oryzias latipes*). Clonal Stem Cell 10, 443–452.

Campbell, K.H.S., Mc Whir, J., Ritchie, W.A., Wilmut, I., 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380, 64–66.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Abel Ponce de León, F., Robl, J.M., 1998. Cloned transgenic calves produced from nonquisent fetal fibroblast. Science 280, 1256–8.

Cibelli, J., Kiessling, A., Cunniff, K., Richards, C., Lanza, R.P., West, M.D., 2001. Somatic Cell Nuclear Transfer in Humans: Pronuclear and Early Embryonic Development. J. Regen. Med. 2, 25–31.

Chen, H., Yi, Y., Chen, M., Yang X., 2010. Studies on the developmental potentiality of cultured cell nuclei of fish. Int. J. Biol. Sci. 6, 192–198.

Chênais, N., Depincé, A., Le Bail, P.Y., Labbé, C., 2014. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of finfish genetic resources. Aquacult. Int. 22, 63–76.

Durruthy-Durruthy, J., Sebastiano, V., Wossidlo, M., Cepeda, D., Cui, J., Grow, E.J., Davila, J., Mall, M., Wong, W.H., Wysocka, J., Au, K.F., Reijo-Pera, R.A., 2016. The primate-specific noncoding RNA HPAT5 regulates pluripotency during human preimplantation development and nuclear reprogramming. Nat. Genet. 48, 44–52.

Evans, M.J., Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156.

Fatira, E., Havelka, M., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T., 2018. Application of interspecific Somatic Cell Nuclear Transfer (iSCNT) in sturgeons and an unexpectedly produced gynogenetic sterlet with homozygous quadruple haploid. Sci. Rep. 8, 5997.

Fatira, E., Havelka, M., Labbé, C., Depincé, A., Pšenička, M., Saito, T., 2019. A newly developed cloning technique in sturgeons; an important step towards recovering endangered species. Sci. Rep. 9: 10453.

Flynn, R.A., Chang, H.Y., 2014. Long noncoding RNAs in cell-fate programming and reprogramming. Cell Stem Cell. 14, 752–761.

Fontana, F., Lanfredi, M., Rossi, R., Bronzi, P., Arlati, G., 1996. Karyotypic characterization of *Acipenser gueldenstaedti* with C-, AgNO3 and fluorescence banding techniques. Ital. J. Zool. 63, 113–118.

Fontana, F. et al., 1998. Karyotypic characterization of the great sturgeon, *Huso huso*, by multiple staining techniques and fluorescent in situ hybridization. Mar. Biol. 132, 495–501.

Fontana, F. et al., 2008. Evidence of hexaploid karyotype in shortnose sturgeon. Genome 51, 113–119.

Gasaryan, K.G., Hung, N.M., Neyfakh, A.A., Ivanenkov, V.V., 1979. Nuclear transplantation in teleost *Misgurnus fossilis* L. Nature 280, 585–587.

Gesner, J., Freyhof, J., Kottelat, M., 2010a. *Acipenser gueldenstaedtii*. The IUCN Red List of Threatened Species: e.T232A13042340, https://doi.org/10.2305/IUCN.UK.20101.RLTS.T232A13042340.en.

Gesner, J., Chebanov, M., Freyhof, J., 2010b. *Huso huso*. The IUCN Red List of Threatened Species 2010: e.T10269A3187455, https://doi.org/10.2305/IUCN.UK.20101.RLTS.T10269A3187455.en.

Gesner, J., Freyhof, J., Kottelat, M., 2010c. *Acipenser ruthenus*. The IUCN Red List of Threatened Species 2010: e.T227A13039007, https://doi.org/10.2305/IUCN.UK.20101.RLTS.T227A13039007.en.

Gómez, M.C., Pope, C.E., Dresser, B.L., 2006. Nuclear transfer in cats and its application. Theriogenology 66, 72–81.

Gómez, M.C. Pope, C.E., Ricks, D.M., Lyons, J., Dumas, C., Dresser, B.L., 2009. Cloning endangered felids using heterospecific donor oocytes and interspecies embryo transfer. Reprod. Fertil. Dev. 21, 76–82.

Grand, L., Bemis, W.E., 1991. Osteology and phylogenetic relationships of fossil and recent paddlefishes (Polyodontidae) with comments on the interrelatioships of Acipenseriformes. J. Vertebr. Paleontol. 11, 11–21.

Guenther, M.G., 2011. Transcriptional control of embryonic and induced pluripotent stem cells. Epigenomics 3, 323–343.

Gurdon, J.B., Wilmut, I., 2011. Nuclear transfer to eggs and oocytes. Cold Spring Harb. Perspect. Biol. 3, a002659.

Hochleithner, M., Gessner, J., 2012. The Sturgeons and Paddlefishes (Acipenseriformes) of the World – Biology and Aquaculture. In: Hochleithner, M., Gessner, J., (Eds.), The Sturgeons and Paddlefishes (Acipenseriformes) of the World, AquaTech Publications, Kitzbühel, Austria, pp. 178–190.

Hongtuo, F., Chingjiang, W., 2001. Nuclear transfer in loach (*Paramisgurnus dabryanu* Sauvage) by cell-to cell electrofusion. Aquac. Res. 32, 267–275.

Hu, G., Niu, F., Humburg, B.A., Liao, K., Bendi, S., Callen, S., Fox, H.S., Buch, S., 2018. Molecular mechanisms of long noncoding RNAs and their role in disease pathogenesis. Oncotarget 9, 18648–18663.

Inoue, K., 2005. Generation of cloned mice by direct nuclear transfer from natural killer T cells. Curr. Biol. 15, 1114–1118.

International Union for Conservation of Nature 2019. The IUCN Red List of Threatened Species, Version 2017–2, www.iucnredlist.org (accessed on 6 September 2019).

Iwamatsu, T., 2004. Stages of normal development in the medaka *Oryzias latipes*. Mech. Dev. 121, 605–618.

Ju, B. S. et al., 2003. Development and gene expression of nuclear transplants generated by transplantation of cultured cell nuclei into non-enucleated eggs in the medaka *Oryzias latipes*. Dev. Growth Differ. 45, 167–174.

Kaftanovskaya, E., Motosugi, N., Kinoshita, M., Ozato, K., Wakamatsu, Y., 2007. Ploidy mosaicism in well-developed nuclear transplants produced by transfer of adult somatic cell nuclei to nonenucleated eggs of medaka (*Oryzias latipes*). Dev. Growth Diff. 49, 691–698.

Kato, Y., Tani, T., Sotomaru, y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H., Tsunoda, Y., 1998. Eight calves cloned from somatic cells of a single adult. Science 282, 2095–2098.

Labbé, C., Robles, V., Herraez, M.P., 2013. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In: Allan, G., Burnell, G., (Eds.) Advances in aquaculture hatchery technology. Woodhead Publishing, Elsevier Academic Press, pp. 76–116.

Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D., Damiani, P., 2000. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning 2, 79–90.

Le Bail, P.Y., Depincé, A., Chênais, N., Mahe, S., Maisse, G., Labbé, C., 2010. Optimization of somatic cell injection in the perspective of nuclear transfer in goldfish. Dev. Biol. 10, 64.

Lee, K.Y., Huang, H., Bensheng, J., Yang, Z., Lin, S., 2002. Cloned zebrafish by nuclear transfer from long-term-cultured cells. NPG 20, 795–799.

Li, Y., Kato, Y., Tsunoda, Y., 2006. Cloned endangered species takin (*Budorcas taxicolor*) by inter-species nuclear transfer and comparison of the blastocyst development with yak (*Bos grunniens*) and bovine. Mol. Reprod. Dev. 73, 189–195.

Liu, T.M., Yu, X.M., Ye, Y.Z., Zhou, J.F., Wang, Z.W., Tong, J.G., Wu, C.J., 2002. Factors affecting the efficiency of somatic cell nuclear transplantation in the fish embryo. J. Exp. Zool. 293, 719–725.

Liu, J., Sun, Y.H., Wang, Y.W., Wang, N., Pei, D.S., Wang, Y.P., Hu, W., Zhu, Z.Y., 2008. Identification of differential transcript profiles between mutual crossbred embryos of zebrafish (*Danio rerio*) and Chinese rare minnow (*Gobiocypris rarus*) by cDNA-AFLP. Theriogenology 70, 1525–1535.

Loewer, S., Gabili, M.N., Guttman, M., Loh, Y.H., Thomas, K., Park, I.H., Garber, M., Curran, M., Onden, T., Agarwal, S., Manos, P.D., Datta, S., Lander, E.S., Schlaeger, T.M., Daley, G.Q., Rinn, J.L., 2010. Nat. Genet. 42, 1113–1117.

Loi, P., Ptak, G., Barboni, B., Fulka, J.Jr., Cappai, P., Clinton, M., 2001. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortemsomatic cells. Nat. Biotechnol. 19, 962–964.

Loi, P., Galli, C., Ptak, G., 2007. Cloning of endangered mammalian species: any progress? Trends Biotechnol. 25, 195–200.

Lubzens, E., Young, G., Bobe, J., Cerda, J., 2010. Oogenesis in teleosts: How fish eggs are formed. Gen. Comp. Endocr. 165, 367–389.

Ludwig, A., Makowiecki, D., Benecke, N., 2009. Further evidence of trans-Atlantic colonization of Western Europe by American Atlantic sturgeons. Archaeofauna 18, 185–192.

Luo, D., Hu, W., Chen, S., Xiao, Y., Sun, Y., Zhu, Z., 2009. Identification of differentially expressed genes between cloned and zygote-developing zebrafish (*Danio rerio*) embryos at the dome stage using suppression subtractive hybridization. Biol. Reprod. 80, 674–684.

Luo, D.J., Hu, W., Chen, S.P., Zhu, Z.Y., 2011. Critical developmental stages for the efficiency of somatic cell nuclear transfer in zebrafish. Int. J. Biol. Sci. 7, 476–486.

Meissner, A., Jaenisch, R., 2006. Mammalian nuclear transfer. Dev. Dynam. 235, 2460–2469.

Mc Grath, J., Solter, D., 1983. Nuclear transplantation in the mouse embryo by microsurgery and cell-fusion. Science 220, 1300–1302.

Mhanni, A.A., Mc Gowan, R.A., 2004. Global changes in genomic methylation levels during early development of the zebrafish embryo. Dev. Genes Evol. 214, 412–417.

Moritz, C., Labbé, C., 2008. Cryopreservation of goldfish fins and optimization for field scale cryobanking. Cryobiology 56, 181–188.

Niwa, K., Ladygina, T., Kinoshita, M., Ozato, K., Wakamatsu, Y., 1999. Transplantation of blastula nuclei to non-enucleated eggs in the medaka, *Oryzias Latipes*. Dev. Growth Differ. 41, 163–172.

Oback, B., 2008. Climbing mount efficiency-small steps, not giant leaps towards higher cloning success in farm animals. Reprod. Domest. Anim. 43, 407–416.

Ogura, A., Inoue, K., Wakayama, T., 2013. Recent advancements in cloning by somatic cell nuclear transfer. Philos. Trans. R. Soc. Lond. B 368, 20110329.

Oh, H.J., Kim, M.K., Jang, G., Kim, H.J., Hong, S.G., Park, J.E., 2008. Cloning endangered gray wolves (*Canis lupus*) from somatic cells collected postmortem. Theriogenology 70, 638–647.

Pei, D.S., Sun, Y.H., Chen, S.P., Wang, Y.P., Hu, W., Zhu, Z.Y., 2007. Identification of differentially expressed genes from the cross-subfamily cloned embryos derived from zebrafish nuclei and rare minnow enucleated eggs. Theriogenology 68, 1282–1291.

Pei, D.S., Sun, Y.H., Chen, C.H., Chen, S.P., Wang, Y.P., Hu, W., Zhu, Z.Y., 2008. Identification and characterization of a novel gene differentially expressed in zebrafish cross-subfamily cloned embryos. BMC Dev. Biol. 8, 29.

Pei, D.S., Sun, Y.H., Zhu, Z.Y., 2009. Identification of a novel gene K23 over-expressed in fish cross-subfamily cloned embryos. Mol. Biol. Reprod. 36, 1375–1380.

Perez-Camps, M., Cardona-Costa, J., Garcia-Ximenez, F., 2010. Transplantation of adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid-blastula transition embryos. Zebrafish 7, 215–218.

Rideout, W.M., Eggan, K., Jaenisch, R., 2001. Nuclear cloning and epigenetic reprogramming of the genome. Science 293, 1093–1098.

Rouillon, C., Depincé, A., Chênais, N., Le Bail, P.Y., Labbé, C., 2019. Somatic cell nuclear transfer in non-enucleated goldfish oocytes: understanding DNA fate during meiosis resumption and first cellular division. Sci. Rep. 9:12462.

Saito, T., Pšenička, M., Goto, R., Adachi, S., Inoue, K., Arai, K., Yamaha, E., 2014. The origin and migration of primordial germ cells in sturgeon. PLoS ONE 9, e86861.

Siripattarapravat, K., Pinmee, B., Venta, P.J., Chang, C.C., Cibelli, J.B., 2009. Somatic cell nuclear transfer in zebrafish. Nat. Methods 6, 733–735.

Siripattarapravat. K., Pinmee, B., Chang, E.A., Munoz, J.D., Kawakami, K., Cibelli, J.B., 2010. The influence of donor nucleus source on the outcome of zebrafish somatic cell nuclear transfer. Int. J. Dev. Biol. 54, 1679–1683.

Shaoyi, Y., Zhongrung, M., Huiyi, Y., Miao, T., Shukhong, L., Guoping, H., Guanshan, L., Lin, G., Guanquin, J., Ruifen, H., Lidong, L., Shiling, X., Leining, Y., Dequan, X., Nianci. Z., 1991. Further investigation on nuclear transplantation in different orders of teleost: the combination of nucleus of tilapia (*Oreochromis nilotica*) and the cytoplasm of loach (*Paramisgurnus dabryanus*). Int. J. Dev. Biol. 35, 429-435.

Solti, L., Crichton, E.G., Loskutoff, N.M., Cseh, S., 2000. Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. Theriogenology 53, 149–162.

Srirattana, K., Imsoonthornruksa, S., Laowtammathron, C., Sangmalee, A., Tunwattana, W., Thongprapai, T., Chaimongkol, C., Ketudat-Cairns, M., Parnpai, R., 2012. Full-term development of gaur-bovine interspecies somatic cell nuclear transfer embryos: Effect of trichostatin A treatment. Cell. Reprogram. 14, 248–257.

St John, J.C., Lloyd, R.E.I., Bowles, E.J., Thomas, E.C., El Shourbagy, S., 2004. The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. Reproduction 127, 631–641.

Sun, F.Z., Hoyland, J., Huang, X., Mason, W., Moor, R.M., 1992. A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development 115, 947–956.

Sun, Y.H., Chen, S.P., Wang, Y.P., Hu, W., Zhu Z.Y., 2005. cytoplasmic impact on cross-genus cloned fish derived from transgenic common carp (*Cyprinus carpio*) nuclei and goldfish (*Carassius auratus*) enucleated eggs. Biology of Reproduction 72, 510–515.

Tagliavini, J., Williot, P., Congiu, L., Chicca, M., Lanfredi, M., Rossi, R., Fontana, F., 1999. Molecular cytogenetic analysis of the karyotype of the European Atlantic sturgeon, *Acipenser sturio*. Heredity 83: 520–525.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 126, 663–676.

Tanaka, D., Takahashi, A., Takai, A., Ohta, H., Ueno, K., 2012. Attempt at cloning high-quality goldfish breed 'Ranchu' by fin-cultured cell nuclear transplantation. Zygote 20, 79–85.

Tung, T.C., Wu, S.C., Tung, Y.Y.F., Yen, S.S., Tu, M., Lu, T.Y., 1963. Nuclear transplantation in fishes. Sci. Sin. XIV: 1244–1245.

Tung, T.C., Tung, Y.F.Y., Luh, T.Y., 1965. Transplantation of nuclei between two subfamilies of teleosts (goldfish-domesticated *Carassius auratus*, and Chinese bitterling- *Rhodeus sinensis*). Acta Zool. Sinica 19, 210–212.

Vajta, G., 2007. Somatic cell nuclear transfer in its first and second decades: successes, setbacks, paradoxes and perspectives. Reprod. Biomed. Online 15, 582–590.

Vasil'ev, V.P., Vasil'eva, E.D., Shedko, S.V., Novomodny, G.V., 2009. Ploidy levels in the kaluga *Huso dauricus* and sakhalin sturgeon *Acipenser mikadoi* (Acipenseridae, Pisces). Dokl. Biol. Sci. 426, 228–231.

Wakamatsu, Y., Ju, B., Pristyaznhyuk, I., Niwa, K., Ladygina, T., Kinoshita, M., Araki, K., Ozato, K., 2001. Fertile and diploid nuclear transplants derived from embryonic cells of a small laboratory fish, medaka (*Oryzias latipes*). Proceedings of the National Academy of Sciences of the United States of America (PNAS) 98, 1071–1076.

Wakamatsu, Y., 2008. Novel method for the nuclear transfer of adult somatic cells in medaka fish (*Oryzias latipes*): Use of diploidized eggs as recipients. Develop. Growth Differ. 50, 427–436.

Wani, N.A., Vettical, B.S., Hong, S.B., 2017. First cloned Bactrian camel (*Camelus bactrianus*) calf produced by interspecies somatic cell nuclear transfer: A step towards preserving the critically endangered wild Bactrian camels. PLoS One 12, e0177800.

Wildt, D.E., 1992. Genetic resource banks for conserving wildlife species: justification, examples and becoming organized on a global basis. Anim. Reprod. Sci. 28, 247–257.

Yan, S.Y., Mao, Z.R., Yang, H.Y., Tu, M.A., Li, S.H., Huang, G.P., Li, G.S., Guo, L., Jin, G.Q., He, R.F., 1991. Further investigation on nuclear transplantation in different orders of teleost: the combination of the nucleus of Tilapia (*Oreochromis nilotica*) and the cytoplasm of Loach (*Paramisgurnus dabryanus*). Int. J. Dev. Biol. 35, 429–435.

Yan, S.Y., 1998. Cloning in Fish: Nucleaocytoplasmic Hybrids. IUBS Educational and Cultural Press, Hong Kong.

Yan, Z.Z., Sun, Z.h., 2000. Embryonic and genetic manipulation in fish. Cell Res. 10, 17–27.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H.S., 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810–813.

Zhu, Z.Y., Sun, Y.H., 2000. Embryonic and genetic manipulation in fish. Cell. Res. 10, 17–27.

CHAPTER 2

APPLICATION OF INTERSPECIFIC SOMATIC CELL NUCLEAR TRANSFER (ISCNT) IN STURGEONS AND AN UNEXPECTEDLY PRODUCED GYNOGENETIC STERLET WITH HOMOZYGOUS QUADRUPLE HAPLOID

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Application of interspecific Somatic Cell Nuclear Transfer (iSCNT) in sturgeons and an unexpectedly produced gynogenetic sterlet with homozygous quadruple haploid

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Somatic cell nuclear transfer (SCNT) is a very promising cloning technique for reconstruction of endangered animals. The aim of the present research is to implement the interspecific SCNT (iSCNT) technique to sturgeon; one fish family bearing some of the most critically endangered species. We transplanted single cells enzymatically isolated from a dissociated fin-fragment of the Russian sturgeon (*Acipenser gueldenstaedtii*) into non-enucleated eggs of the sterlet (*Acipenser ruthenus*), two species bearing different ploidy (4n and 2n, respectively). Up to 12% of the transplanted eggs underwent early development, and one feeding larva (0.5%) was successfully produced. Interestingly, although this transplant displayed tetraploidism (4n) as the donor species, the microsatellite and species-specific analysis showed recipient-exclusive homozygosis without any donor markers. Namely, with regards to this viable larva, host genome duplication occurred twice to form tetraploidism during its early development, probably due to iSCNT manipulation. The importance of this first attempt is to apply iSCNT in sturgeon species, establishing the crucial first steps by adjusting the cloning-methodology in sturgeon's biology. Future improvements in sturgeon's cloning are necessary for providing with great hope in sturgeon's reproduction.

The development of reproductive biotechnology is opening a new window for the conservation of threatened wildlife, as a back up when all other protection policies have failed. In this sense, nuclear transfer, also called cloning, is expected to be a useful tool to preserve species that are nearly extinct or to reconstruct extinct species[1–6]. Interspecific somatic cell nuclear transfer (iSCNT) application to endangered fish species has a great advantage, as the reconstruction of the critically threatened species can be achieved after a single fin-cell is transplanted in the egg-cytoplasmic environment of species whose eggs are easily available in farms. Interspecific cloning using somatic cells has been reported in vulnerable or threatened mammal species like gaur calf, *Bos gaurus*[3,7], European mouflon, *Ovis orientalis*[5], black footed cat, *Felis nigripes*[8] and more recently in Bactrian camel, *Camelus bactrianus*[9]. However, as far as we know, this technique has not been applied to an endangered species. Sturgeons are known as a relict group of species and are amongst the most endangered organisms due to habitat degradation, over-harvesting and illegal fishing for their roe[10,11]. Therefore, it is considered one of the most suitable candidates for cloning in practice. The Acipenseridae family contains species with an interesting specificity; i.e. different ploidy between them. For instance, sterlet, *Acipenser ruthenus*, beluga, *Huso huso* and the European sea sturgeon, *A. sturio* are diploid[12–14] while the Sakhalin, *A. mikadoi* and Russian sturgeon, *A. gueldenstaedtii* are tetraploid[15,16] and the shortnose sturgeon, *A. brevirostrum* is hexaploid[17].

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The principle of somatic cell nuclear transfer (SCNT) is that the differentiated donor cell could be restored to full totipotency when it is transplanted in the prosperous environment of a recipient egg[18]. However, the cloning success is limited in fish, even in model species like zebrafish, *Danio rerio*. Since the first adult cloned zebrafish, obtained by Lee, *et al.*[19] from long-term cultured fibroblast cells, the success of 2% has not been increased using somatic cells. In general, fish SCNT is mostly unsuccessful in producing living adult clones[20–24]. Furthermore, teleost iSCNT yields only poor early embryonic development. This is the case of cloning gynogenetic bighead carp, *Aristichthys nobilis* using gibel carp, *Carassius auratus gibelio* activated eggs that resulted in a success of 27% until blastula stage[25].

In order to perform the cloning technique, some basic steps must first be established. For instance, optimization of donor cell preparation is one crucial step because cell viability is the primary factor for a successful SCNT. Thus, a piece of fin possesses a high regenerative capacity[26] and it is easily available, causing minimum damage to the fish[6,26]. This is especially valuable in the case of critically endangered species like sturgeon. Furthermore, the fin-tissue can be harvested even before sexual maturation of the individual[27] and this is very important for sturgeon species like the beluga whose first maturation usually comes at ~20 years. Another important question for SCNT is whether an extender solution is needed for both egg washing and the working medium. In sturgeon, the eggs need to be washed prior to micromanipulation as they come with coelomic fluid of high viscosity that contains a lot of ovarian somatic and blood cells. To prevent the possibility of transplanting an ovarian somatic cell into the recipient egg, the eggs need to be washed with a physiological saline solution first. During the SCNT procedure, it is convenient that both donor cells and recipient eggs be placed in the same petri dish with the extender solution. Therefore, the medium should not be toxic to any of them, and it must not trigger or enhance egg activation[28]. Just after SCNT, the nuclear transplants (NTs) need to be incubated for a certain period of time before the activation is triggered, so that the donor nucleus can be given the opportunity to equilibrate in the new environment and be reprogrammed[27].

For sturgeon cloning, to use non-enucleated eggs seems to be preferable, because an enucleation of sturgeon eggs can be harmful and requires time and complicated procedures. In addition, putative spontaneous egg-enucleation[22] could result in the normal development of the reconstructed embryo[29]. According to Le Bail, *et al.*[28], transplantation via the micropyle without egg activation seems to improve the efficiency of cloned fish production. However, in this case, a microneedle must be customized for the egg chorion penetration. Finding the correct way of inserting the microneedle, and at which depth within the egg, is a real challenge due to the big size of sturgeon eggs: e.g. sterlet egg, 1.8–2.8 mm; Russian sturgeon egg, 2.8–3.8 mm[30]. Nevertheless, the microinjection position is highly important, as the donor fin-nucleus must be at a favourable place in the host environment in order to be reprogrammed and to produce a cloned sturgeon.

Within the Acipenseridae family, the Russian sturgeon that is classified as critically endangered[11] is the most preferred for caviar consumption[30]. Despite the advantage of a large amount of eggs per kg of body-weight (10,000–15,000), the drawback of the species is the late age at which the first gonad maturation is settled, 10–16 and 8–13 for females and males, respectively[30]. As a recipient species, the sterlet is promising. Indeed, although it is classified as a vulnerable species[11], it is considered to be a model species for Acipenseridae as it is one of the smaller sturgeon species and is easily assessable in fish farms. The most important benefit is that its sexual maturity is reached earlier than other sturgeon species, at age 5–8 and 3–5, for female and male, respectively. Additionally, spawning takes place in cycles of 1–2 years in both sexes, which is much more often than other species within the same family[30].

Overall, the SCNT methodology is a very delicate procedure that requires optimization of many experimental conditions with precise techniques and skilful manipulations. Therefore, the aim of this study was to test the possibility whether the iSCNT can be applied to real endangered species. For this purpose, individuals from Russian sturgeon were used as somatic cell donor species, whereas individuals from sterlet were used as recipient egg providers. The crucial steps of SCNT were examined with these sturgeon species in practice, using shallow-injection of Russian sturgeon's single fin-cells into the animal pole of sterlet's non-enucleated and non-activated eggs.

Results

Extender solution. Four extender solutions and filtrated water (FW) were tested to find the efficient solution that is able to maintain the recipient eggs inactivated (meiosis II stage) during micromanipulation. All four extender solutions tested gave fairly high fertilization rates (Fig. 1). Whatever the extender solution, the incubation time did not significantly alter the egg ability to be fertilized afterwards. Significant differences ($F = 13.660$, $p < 0.001$) in fertilization rate were detected among extender solutions. All extender solutions showed higher fertilization rates compared to the FW group (Tukey's HSD $p < 0.01$) but no statistically significant differences were detected among them (Tukey's HSD $p > 0.05$). It indicates that some activation occurred when the eggs were manipulated in this medium, and that it affected their ability to be fertilized. From these results, and in order to standardize our working solution, we chose to use Persian sturgeon artificial coelomic fluid (PSACF) and 30 min of incubation for sturgeon-SCNT. Thus, it showed the highest fertilization rates and less variable results after 30 min of incubation (Fig. 1), which is the duration of the SCNT micromanipulation procedure. In fact, there was no sign of egg-activation (no elevation of chorion in sturgeon eggs) while they were kept in PSACF during the whole SCNT procedure.

Somatic cell nuclear transfer. After the dissociation of fin-tissue from albino sterlet or Russian sturgeon by trypsinization, the cell density was $270,000 \pm 8,000$ or $275,000 \pm 10,000$ cells/mL, respectively, with an average cell viability of $95 \pm 5\%$ (5 replicates each). Using albino sterlet fin-cells, 129 sterlet eggs reconstructed, from which 13 NTs (10.1%) exhibited initial cleavages and reached the blastula stage. Only 4 embryos (3.1%) reached the gastrula stage and stopped development (Table 1). Using Russian sturgeon fin-cells, in total 210 sterlet eggs submitted to iSCNT and 14 NTs (6.7%) showed sequential cleavages with a normal pattern up to the blastula stage.

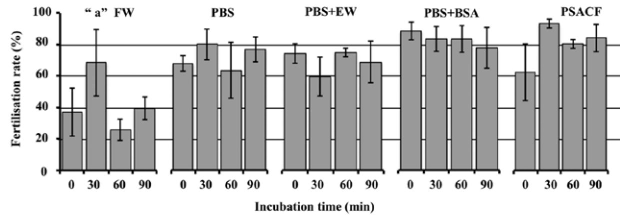

Figure 1. Comparison of extender solutions for egg preservation. Five solutions: filtrated water (FW), phosphate buffered saline (PBS), PBS with egg-white (EW), PBS with bovine serum albumin (BSA) and Persian sturgeon artificial coelomic fluid (PSACF) were tested for their ability to maintain the eggs inactivated. Eggs were washed and incubated for different duration (0 min, 30 min, 60 min or 90 min) in their respective solution. Each value represents the mean ± s.d. fertilization rate (%) from 3 different spawns. Letter “a”: Significant difference ($p < 0.001$) of control (FW) is indicated by Tukey’s HSD.

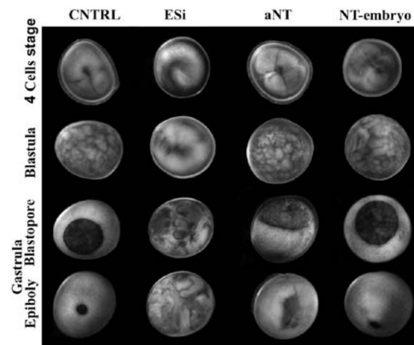

Figure 2. After iSCNT, comparison of early development between a fertilized sterlet embryo from the control group (CNTRL), an extender injected sterlet egg (ESi), an NT-embryo that stopped development at a later stage (2/3 epiboly in gastrula stage)-arrested NT (aNT) and the NT-embryo that surpassed the gastrula stage (completion of epiboly). The aNT-embryo exhibited normal cleavage up to gastrula stage and formation of the blastopore but didn’t reach the epiboly. The NT-embryo exhibited normal cleavages, blastopore formation, and the completion of the gastrulation with a similar pattern to the CNTRL.

Experimental group	Total eggs	Blastula (%)	Gastrula (%)	Neurula (%)	Hatching (%)	Feeding (%)
Albino-NTs	129	13 (10.1)	4 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)
Russian-NTs	210	14 (6.7)	10 (4.8)	3 (1.4)	1 (0.5)	1 (0.5)
Fertilized CNTRL	450	251 (55.8)	243 (54.0)	230 (51.1)	230 (51.1)	230 (51.1)
Extender-injected CNTRL	50	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Table 1. Developing rates of reconstructed embryos after albino sterlet fin-cells transfer (Albino-NTs) or Russian sturgeon fin-cells transfer (Russian-NTs) into sterlet eggs and CNTRLs at each stage.

Among them, some embryos stopped their development and only 10 NTs (4.8%) reached the gastrula stage, and exhibited the blastopore on the margin between the animal and the vegetal hemisphere (Fig. 2; see aNT). While 7 embryos stopped their development and blastomeres were broken down on the course of gastrulation, 3 (1.4%) completed this step and reached the neurula stage. Finally, 1 NT-embryo (0.5%) successfully formed the neural fold and somites (Fig. 3A) and entered the somatogenesis stage that displayed swelled-shape heart (Fig. 3B; see Supplementary Videos S1 and S2). The NT successfully hatched out and fed (Table 1; Fig. 3C; see Supplementary Video S3) while its pigmentation pattern was similar to that of the sterlet control (CNTRL) (Fig. 3D). After three

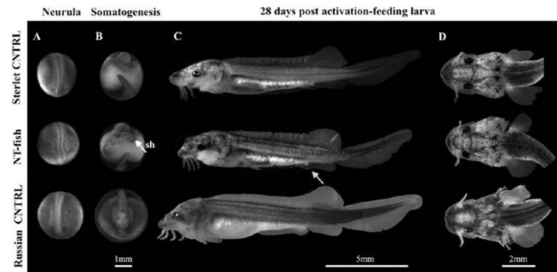

Figure 3. Comparison of the development of sterlet CNTRL, NT-fish obtained after iSCNT and Russian CNTRL from the neurula stage. (**A**) At neurula stage the NT-embryo showed a comparatively normal embryonic development, although its shape seemed to be slightly distorted compared to that of CNTRLs, as the neural fold is bent. (**B**) In the somatogenesis period, the heart of NT-fish showed swelled shape (swelled heart, sh), compared to sterlet and Russian CNTRLs. (**C**) The sterlet and Russian CNTRLs, and the NT-larva at 28 days post activation. At this stage, the slightly distorted body of NT fish observed at neurula stage becomes inconspicuous, providing normal swimming performance. The swelled heart observed in somatogenesis stage in the NT-fish becomes as normal as seen in Russian and sterlet CNTRLs. The stomach of the NT-larva is filled with food (orange arrow). (**D**) The pigmentation pattern of NT-fish is similar to that of sterlet CNTRL.

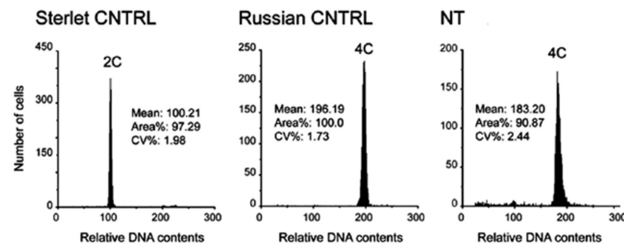

Figure 4. Relative ploidy levels of one sterlet control (CNTRL), one Russian control (CNTRL) and of the 28 days old NT that obtained after iSCNT. Two ploidy levels were observed: 2n in sterlet CNTRL, 4n in Russian CNTRL and 4n in NT-larva. The profiles shown for the CNTRL both sterlet and Russian are similar for all the tested fish.

times washing with PSACF and 30 min incubation, more than 50% CNTRL embryos fertilized (n = 251) and developed normally and reached the feeding stage (n = 230) (Table 1). All extender solution-injected CNTRLs (n = 50) did not exhibit development as no somatic cell was injected (Table 1; Fig. 2; see ESi).

Flow cytometry and Genotyping. The ploidy of sterlet CNTRL, Russian CNTRL and of the hatched NT-fish obtained after iSCNT were analysed by flow cytometry. The relative ploidy levels of all sterlet CNTRLs and Russian CNTRLs showed 2n and 4n, respectively (Fig. 4). The cells from the NT-fish at 28 days post activation showed 4n as Russian CNTRL (Fig. 4).

Using sterlet positive primer pair 247_ARp + 247_uni, we obtained amplification of a 247 bp fragment in NT-fish, sterlet-recipient, sterlet male, and sterlet CNTRL embryos, while no amplification in Russian-donor, as expected (Fig. 5). On the contrary, no amplification in NT-fish, sterlet recipient male, and sterlet CNTRLs, but amplification of a 247 bp fragment in Russian-donor, was observed when using sterlet negative primer pair 247_ARn + 247_uni (Fig. 5). It clearly showed that NT-fish contains only sterlet genome. It was consistent with results of microsatellite genotyping, which confirmed no presence of any allele specific to Russian sturgeon in genotypes of NT-fish (Table 2). More interestingly, NT-fish was fully homozygous at all genotyped loci, including loci where sterlet recipient was heterozygote (Table 2).

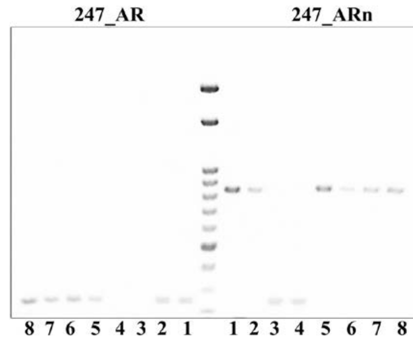

Figure 5. Results of amplification of all samples after iSCNT by sterlet specific primer 247_AR and sterlet negative primer 247_ARn. The most important samples (NT-fish and Russian sturgeon donor) were amplified twice in 2 independent replicates. 1–2 = NT-fish; 3–4 = Russian sturgeon donor; 5 = sterlet recipient; 6 = sterlet male; 7–8 = controls. 750 bp band by 247_ARn primer is not specific and does not affect results.

	Afu_19		**Afu_68**					**Spl_107**				
NT-fish	158					232				302		
NT-fish	158					232				302		
Russian-donor		167	148	164	212		248	286		302		318
Russian-donor		167	148	164	212		248	286		302		318
Sterlet-recipient	158					232				302	310	
Sterlet-male	158								298		310	
CNTRL 1	158									302	310	
CNTRL 2	158								298		310	
	Aox_27				**Spl_163**			**Spl_173**				
NT-fish		138			214			238				
NT-fish		138			214			238				
Russian-donor	134		154	158		222			240		268	
Russian-donor	134		154	158		222			240		268	
Sterlet-recipient		138			214		226	238		254		
Sterlet-male		138				222		238		254		
CNTRL 1		138				222	226	238		254		
CNTRL 2		138				222	226	238		254		
	Aox_45											
NT-fish			156									
NT-fish			156									
Russian-donor	134	137		159	184							
Russian-donor	134	137		159	184							
Sterlet-recipient			156	159								
Sterlet-male			156	159								
CNTRL 1			156	159								
CNTRL 2			156	159								

Table 2. Microsatellite genotyping results. The NT-fish obtained after iSCNT does not possess any allele specific for Russian-donor genome. NT-fish is homozygous at all loci, including loci, where sterlet female recipient is heterozygote. NT-fish and Russian-donor were analysed in two independent replicates. CNTRL 1 and CNTRL 2 are sterlet control embryos at the same age of NT-fish that obtained after *in vitro* fertilization of Sterlet-recipient and Sterlet-male.

Discussion

Our experiment is utilizing the interspecific cloning technique in a real endangered species. Using the Russian sturgeon as the donor fin-cell species and the sterlet as the recipient egg species we could establish the basic steps of the nuclear transfer technique. We succeeded to obtain 12% NT early development (i.e. 6.7% NT development in comparison to 56% CNTRL development). Such survival provides great hope for future success in sturgeon-iSCNT.

We decided to work as Siripattarapravat, *et al.*[23] and Le Bail, *et al.*[28] on non-activated eggs. Indeed, postponing the egg activation after nucleus injection was shown to increase the NT success when compared to immediate activation[28]. We believe that donor nucleus exposure to the non-activated egg factors is favourable to reprogramming. One reason is that non-activated eggs have high mitosis-promoting factor (MPF) activity[31] that is likely favourable to donor cell reprogramming. It cannot be excluded either that the resting time that we provided to the clone before triggering meiosis resumption and first mitosis allowed some recovery from the mechanical disturbance induced by the nucleus injection. This put us at some distance from most of the other works done in fish that have used activated recipients[19,25].

In the present research, we used non-enucleated sterlet eggs according to Wakamatsu,[22] and Le Bail, *et al.*[28]. It has been suggested that there is an unknown mechanism that allows the female nuclear DNA to be lost after nuclear transfer in medaka, *Oryzias latipes*[32] or in goldfish, *Carassius auratus*[27]. Indeed, Gasaryan, *et al.*[33] observed high percentage of diploids from donor origin in the non-enucleated transplants (up to 70%) in loach, *Misgurnus fossilis*. One advantage of skipping the enucleation step is that almost twice as many eggs can be treated in the same experiment[27]. When comparing the use of enucleated and non-enucleated eggs from goldfish and bitterling, *Rhodeus sinensis*, no significant difference has been shown in the development of NTs embryos and hatchlings after transplantation of donor embryonic cell[34]. Also, no significant difference has been observed in early development of NTs after transplantation of somatic cells from gynogenetic bighead carp into enucleated or non-enucleated gibel carp eggs[25]. In the study of Liu, *et al.*[25], the non-irradiated group exhibited the same development (27.27%) as the treated group (25.71%) at the blastula stage. In our work, we preferred to avoid enucleation because of the risk of damage or losses of the maternal materials (i.e. proteins, mRNAs, mitochondria) that are necessary to support the developing embryo. Since SCNT is a complicate procedure and the developmental rates of the resulted transplants are at stake, we found it preferable to use non-enucleated eggs because they have the same or better result than eggs after mechanical enucleation.

The first and perhaps the most crucial step for a successful SCNT is the use of a suitable extender solution that will maintain the ovulated eggs in metaphase II stage, and that will not be damaging to the fin cells during the micromanipulation. In some fish species, coelomic fluid is used for SCNT. For instance, salmonid ovarian fluid has proved to be suitable to maintain zebrafish[31,35,36] and goldfish[37] eggs in an inactivated state. Our results demonstrated that sturgeon eggs can remain inactivated even though they are incubated into a saline solution. This is quite advantageous in our species, because sturgeon coelomic fluid quality varies among females. Additionally, besides the blood vessels and follicular cells that it contains, its density is so high that it prevents the visualization of the donor cell and its aspiration through the microneedle. Therefore, sturgeon coelomic fluid is not a good candidate to be used in iSCNT. In order to standardize our cloning technique, we chose to use PSACF because our results demonstrated that the egg ability to be fertilized afterwards remained high during the experimental timeline. We also chose to incubate our transplants for 30 min after nuclear transfer before activation, because it has been reported that incubation of eggs after transplantation for a certain period improves the developing rate of clones, probably by prompting the reprogramming of the donor nuclei[23,28]. According to Le Bail, *et al.*[28], hatching stages of goldfish clones were reached only when the nucleus was incubated for at least 30 min prior to egg activation. In sturgeon-iSCNT, such a 30 min- incubation is favourable as this time facilitates the micromanipulation of such a large egg (~20 nuclear transfers).

It was striking to observe that all sturgeon NT-embryos that showed initial cleavage furrows continued development through the mid-blastula stage, and that many of them developed up to the gastrula stage. This result is in contrast with previous studies showing that a high percentage of the NT face difficulties to overcome the mid-blastula stage[22–24,28,38]. The later has been reported to be the stage at which the mitotic checkpoints are established[39], and at which the embryonic genome is activated[40]. Failure to develop further than the mid-blastula stage is inferred to the incomplete epigenetic resetting of the donor nucleus in the egg environment that results in inaccurate zygotic genes expression[24]. Before this stage, embryos develop with maternally supplied factors[41,42]. We noticed that in sturgeon-SCNT, the critical embryonic stage of the NTs seems to be the completion of gastrula. However, we cannot give an efficient explanation for this result since we lack information concerning the stage at which the embryonic genome is activated in our species and when it could affect the developing embryo.

From our results and from previous studies in fish, the SCNT technique doesn't give very hopeful results. The percentage to obtain clone animals even in model fish species varies and is generally low as it is for sturgeon. Cloning success in obtaining embryos after non-enucleation of goldfish egg is 17%[28], after enucleation of zebrafish egg the cloning success in obtaining adult individuals is 2%[19]. In addition, cloning success in medaka after transplantation of fresh harvested fin-cells into diploidized eggs gave 2.7%[43]. Liu, *et al.*[25] discussed that crossing species with different ploidy is an obstacle for embryonic development and this could be a reason of Russian-NT's low success in our study. Even though we are aware that hybridization in Acipenseridae family is high, we do not have information about fertilization rate using sterlet egg (1n) and Russian sturgeon sperm (2n), because the studies on sturgeon are using combination of parents with the same ploidy level. In addition, we still lack sufficient information to explain the difference in the degree of development after iSCNT in sturgeon from the other fish species.

Although we know, from our experience, that the sturgeon embryos display high variability of developmental speed, even in the same batch of eggs that were fertilized simultaneously, we cannot conceal that after iSCNT some NT-embryos and the resulted NT-larva, exhibited slightly faster development than the CNTRL mainly after the blastula stage. These Russian-NTs developed faster than the CNTRL and with a difference of 2 hours. However,

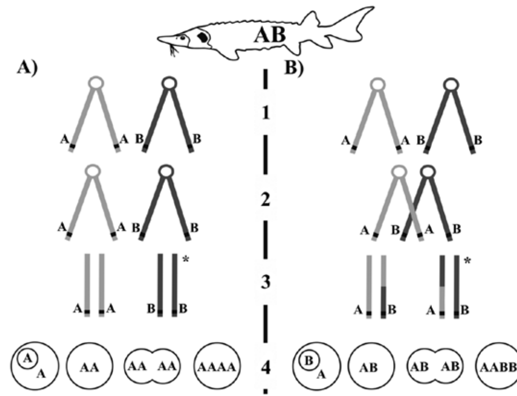

Figure 6. Scheme of meiosis and suggested disturbance in subsequent early embryonic development as possible explanation of NT-fish origin after iSCNT. Sterlet recipient is heterozygote at given locus (AB). (**A**) No crossing over between homologs chromosomes in meiosis followed by retention of 2nd polar body and suppression of first mitotic cleavage results in homozygote individual; (**B**) if crossing over occurs, NT-fish cannot be homozygote under same scenario of post-meiotic development 1 = bivalent of two homologues chromosomes with replicated chromatids; 2 = crossing over between non-sister chromatids occurs in (**B**), while no crossing over in (**A**); 3 = homologues chromosome segregation (anaphase I); *become first polar body. This is followed by standard meiosis II, but second polar body is not released. Thus oocyte is diploid before mitotic cleavage starts; 4 = retention of second polar body and suppression of first mitotic cleavage result in tetraploid homozygote (AAAA) if there is no crossing over in meiosis I - (**A**), but in heterozygote tetraploid (AABB) if crossing over occurs (**B**).

the NT-larva developed faster than CNTRL from the first cleavage until the beginning of the neurula stage, which synchronized with the CNTRL's speed. According to Newport and Kirschner[44], the quicker development of the NT-larva comparing to CNTRL can be explained by the higher NT's ploidy level (double than CNTRL). These researchers demonstrated that *Xenopus* embryos of higher ploidy underwent the MBT at correspondingly earlier times than diploid embryos used as controls. However, the faster development of some NT-embryos does not mean that all of them displayed the same DNA pattern of NT-larva. Thus, some NT-embryos may be real clones, exhibiting the ploidy of Russian sturgeon (4n) or even chimeras, exhibiting both genomes due to fusion of Russian somatic cell with the sterlet egg (4n + 1n = 5n).

Embryonic development of sturgeon species is very similar in pattern of cleavage as well as the morphology of embryo. The larvae of most sturgeon species can be unambiguously identified only by appropriate molecular markers[45]. To uncover, whether NT-fish contains genome of Russian sturgeon donor, we accommodated recently developed nuclear DNA markers[46]. The results of molecular genotyping clearly showed that NT-fish contains only sterlet genome. Thus, any contribution of Russian sturgeon donor to NT-fish, including chimerism, can be excluded.

Two hypotheses can explain this lack of donor contribution while the occurrence of tetraploidism. The first is that there would have been retention of the second polar body and successive suppression of first mitotic cleavage. This would mean that the transplant underwent both events that are more often triggered separately in fish for biotechnological purposes: triploidisation by retention of the second polar body, and diploidisation by preventing the first mitosis. The nucleus of a mature sturgeon egg is at metaphase II stage[47], and any kind of physical or chemical shock applied during meiosis II in fish eggs can prevent the extrusion of the second polar body, while still allowing chromosomal division[48]. Microinjection might work as a physical shock. This scenario, however, is the less probable due to the full homozygosity of NT-fish at analysed loci. NT-fish would be homozygous at loci where sterlet-recipient was heterozygote only if there were no crossovers between the locus and its centromere (Fig. 6A). If the crossing happened, the NT-fish could not be fully homozygote (Fig. 6B).

The second hypothesis, that stands more probable, is the suppression of the first and second mitotic cleavage. Under this scenario, meiosis normally occurred and the second polar body was released after egg activation by injection resulting in the haploid egg. Spontaneous suppression of 1st cleavage resulted in 2n, and another suppression of the following cleavage in 4n cell (Fig. 7). In their striking work on early development in zebrafish, Yabe, *et al.*[49] showed that when the pair of centrioles brought by the spermatozoa was altered or absent, the first mitosis was inhibited although genome duplication had occurred. This produced a first set of whole genome duplication. These authors also showed that after the first mitosis following normal fertilization, the lack

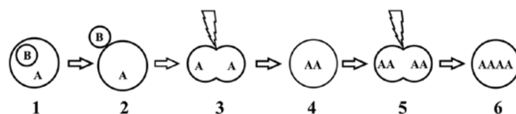

Figure 7. Disturbance in early embryonic development as possible explanation of NT-fish origin after iSCNT. Meiosis occurs normally and second polar body (B) is released after activation of the oocyte (1–2). Whether crossing over occurs or not is not relevant for this scenario because oocyte is haploid before first mitotic cleavage. 3 = suppression of first mitotic cleavage results in diploid homozygote (AA) cell – 4; 5 = suppression of subsequent (second) mitotic cleavage of diploid cell (4) results in tetraploid homozygote (AAAA) cell – 6. This is followed in common embryonic development giving tetraploid, fully homozygote individual.

of specific maternal factors involved in centrosome duplication induced transient defects in cellular cleavage, leading to genome doubling in these blastomeres. Interestingly, these blastomeres could be rescued and resume normal cleavage on subsequent mitosis. A parallel situation between this work and our observations on sturgeon cannot be excluded. In our case, the centrosomes brought by the donor cell may have failed to sustain the first mitosis, and duplication of the haploid maternal genome may have then occurred thanks to some sham fertilization signal brought by the SCNT procedure. Next, the medium injected with the donor cell may have transiently diluted the maternal factors necessary for the second cleavage, resulting in a second round of genome duplication without cell division, followed by normal mitosis resumption. The creation of a 4n individual from unfertilized egg of 2n female is a very interesting result and our data are all relevant with the hypothesis that the donor cell-injection provoked a big stress in the egg, causing gynogenesis.

To the best of our knowledge, the present work is the first report of SCNT among sturgeons. The early development that we have observed proves that SCNT is applicable to ancient chondrosteans as it has been applied in modern teleosteans species[21,25]. It is an easy and cheap technique that can be performed by any laboratory with microinjection experts, as far as the sturgeon-eggs and the fin-tissue can be provided. As a side effect of our iSCNT experiments, unusual disruption in early embryogenesis resulted in fully homozygous tetrahaploid, an event that has not been observed in any sturgeon, or teleostean fish or even vertebrate before. This may have unraveled some mechanism involved in polyploidisation in sturgeon species. Even though we did not succeed to produce any true sturgeon clone, we have established several steps that pave the way for future development of iSCNT in sturgeon in the near future. The use of the cytoplasmic environment from an easy-reared fish like the sterlet with small reproductive maturation can regenerate sturgeons with high value for conservation and management like are Russian sturgeon and beluga.

Methods

Ethics. The transplantation experiments took place at the Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in České Budějovice, Czech Republic. All experimental procedures were performed in accordance with national and institutional guidelines on animal experimentation and care. This study was approved by the Animal Research Committee of the University of South Bohemia in České Budějovice. Fish were maintained according to the principles of animal welfare and principles of laboratory animal care based on the Guidelines for the Use of Animals in Research.

Fish and gamete collection. In order to perform the SCNT technique we used non-sexually matured albino sterlet (2–3 years old) or Russian sturgeon (1–2 years old) as fin-donor species and matured sterlet females (5–6 years old) as egg-recipient species. To perform *in vitro* fertilization of the CNTRL group sperm was collected from matured male sterlet individuals (5–6 years old). Ovulation and spermiation were induced by an intramuscular injection of the gonadotropin-releasing hormone analogue (des-gly10[d-Ala6]-LHRH; Sigma). Eggs and sperm were stripped and fertilized one day after injection. In order to induce ovulation, the sterlet females were injected by a single intramuscular injection of carp pituitary homogenized extract (CPE) at a dose of 5 mg/kg body weight (b.w.) in two steps: first with 0.5 mg/kg b.w. and second with 4.5 mg/kg b.w., 12 h after the first injection. Egg collection was performed 18–20 h after the second injection. In order to induce spermiation, the sterlet males were injected once (4 mg/kg b.w.) with CPE and sperm was collected 48 h after hormonal injection by use of a catheter from the urogenital papilla, transferred to a separate cell culture container (250 mL), and stored at 4°C until sampling (1–3 h).

Examination of the extender solution suitable for egg manipulation. Five solutions were tested as follows: phosphate buffered saline (PBS; SIGMA-ALDRICH®), PBS + 1% bovine serum albumin (BSA; SIGMA-ALDRICH®), PBS + 1% egg white, PSACF[50] and FW. According to Sohrabnezhad, *et al.*[50] PSACF was formulated as 80 mM NaCl, 3.96 mM KCl, 0.78 mM $MgSO_4.7H_2O$, 0.26 mM $CaCl_2$, 2.42 mM glucose, 1 g bovine serum albumin, 20 mM $NaHCO_3$, 20 mM Hepes buffer (4–2-hydroxy-ethyl-1-piperazineethanesulfonic acid), the osmotic pressure was adjusted to 250 mOsm/L and the final pH of the medium was adjusted to 7.5 using 1 N, NaOH. All solutions were kept at 15 °C during experimentation. Three replicates (eggs from 3 sterlet individuals) were performed for each tested group. For each replicate, 17 to 60 eggs were used for each test. For each tested extender, the eggs were washed three times for five minutes in order to remove debris and somatic cells. Then, eggs were incubated for 0, 30, 60, or 90 min at 15 °C in the tested extenders. *In vitro* fertilization was conducted with sterlet fresh-stripped sperm in order to monitor egg quality after incubation. For fertilization, extender

solutions were removed completely, 20 μL sperm were added to each dish, and activated with FW. Fertilization rate was assessed after 8 h (mid-blastula stage) at 15 °C. Differences between extender solutions were analysed using one-way ANOVA test. To determine significant pair-wise differences between treatments Tukey's HSD post-hoc test was applied.

Donor fin-cells preparation. A small piece (0.5 cm^2) of albino sterlet or Russian sturgeon caudal fin was clipped using sterile scissors. The fin fragment was washed with PBS under gentle shaking for one min to remove mucus. One-third of the fin was stored in 96% ethanol for DNA extraction, and the remaining piece was kept for preparation of the donor cells. The fin-tissue was dissociated in 0.5% Trypsin (Gibco® Life Technologies; ThermoFisher SCIENTIFIC) in PBS for 2 h at 15 °C with gentle shaking (TUBE REVOLVER; Thermo SCIENTIFIC). Dissociated cells were filtrated using a 50 μm pore size filter (CellTrics®) and centrifuged at 800xg for 10 min at 15 °C. The cells were then washed twice with PBS containing 0.05% DNase and the last pellet was suspended in 150 μL PBS. Cell viability and concentration were assessed using a haemocytometer (Bright-Line™ Hemacytometer; Hausser Scientific) after Trypan blue staining (SIGMA-ALDRICH®). The cells were kept at 4 °C until use, no longer than 8 h.

Nuclear transfer. After washing with PSACF three times to remove the debris and somatic cells, eggs were placed in a 6 cm^2 petri dish filled with the PSACF at 15 °C. The SCNT was conducted by a hydraulic injector (Cell-Tram Oil; Eppendorf, Germany) connected to a micromanipulator (MO-152; Narishige, Japan) under a stereomicroscope (Leica M165 FC.) according to Le Bail, *et al.*[28]. An egg was fixed on a glass capillary holder (inner diameter: 0.7–0.8 mm) so that the animal pole faced the transfer needle, and a small amount (5 μL) of the fin-cell suspension (270 ± 4 or 275 ± 10 cells/μL for albino sterlet or Russian sturgeon, respectively) was dropped near the egg. Then, a single fin-cell was gently aspirated into the hand-made microcapillary (inner diameter: 25–28 μm) and was introduced in the animal pole of the egg, where the micropyles are located. After SCNT using fin-cells from albino sterlet (4 experiments) or Russian sturgeon (6 experiments), the transplants (n = 129 or 210, respectively) were incubated in the PSACF (15 °C) for 30 min and then were activated with FW. Simultaneously, fertilization of the CNTRL group with freshly stripped sterlet sperm was performed to produce CNTRL sterlet embryos (n = 450). For a negative CNTRL, we used eggs injected with the extender solution without a fin-cell, and after 30 min incubation they activated with water (n = 50).

Cultivation of the embryos. After activation of the nuclear-transplants (NTs) and fertilization of the CNTRL group, all eggs were treated with 0.1% tannic acid (SIGMA-ALDRICH®) for 10 min to remove the surface stickiness. After washing the embryos three times with water, embryos were cultured in FW containing 0.01% penicillin and 0.01% streptomycin for three days at 15 °C. Forceps were used to remove the outer layers of chorion at 10 h post-activation. The inner layer of the chorion was removed at 5–6 days post activation. First feeding started after yolk resorption, 20 days after fertilization. The freshwater annelid worm *tubifex* sp. was given twice per day at 8:00am and 16:00 pm. Tanks were cleaned twice per day 2 h after the meal.

Ploidy assessment. Fragments of caudal fins from five adult sterlet and two Russian sturgeons, and from the 28-days-old feeding larvae (CNTRL and NT-fish) were used for ploidy assessment. The tissues were minced in extraction buffer and then stained with 4-6-diamino-2 phenylindole dihydrochloride (CyStain DNA 2step kit; Partec GmbH). Ploidy of these samples was determined by flow cytometry (Ploidy Analyser; Partec).

Molecular genotyping. Fin clips from both cell-donor (Russian sturgeon) and egg-recipient females (sterlet) were collected and fixed, as mentioned above. Genomic DNA was extracted using a DNA extraction kit according to manufacturer's instructions (GenElute Mammalian Genomic DNA Miniprep Kit; SIGMA-ALDRICH®). Six samples, DNA from NT-fish, sterlet-recipient, sterlet-male, Russian-donor and 2 sterlet CNTRL embryos were analysed.

Samples were tested by sterlet positive primer pair 247_AR + 247_uni and subsequently by sterlet negative primer pair 247_ARn + 247_uni as described in Havelka, *et al.*[46]. If a sample contains only sterlet DNA, the 247 bp fragment is amplified only by sterlet positive primer pair. If a sample contains DNA of other sturgeon species, the 247 bp fragment is amplified only by sterlet negative primer pair. Finally, if a sample contains DNA of sterlet and other sturgeon species (e.g. sample is hybrid or chimera) 247 bp fragment is amplified by both primer pairs in two independent reactions. Briefly, PCR reactions were performed in a total volume of 25 μL containing 0.25 μM of each primer, 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 800 μM dNTP, 2.5U Taq-Purple DNA polymerase, and 25 ng of DNA template under following cycling conditions: 95 °C for 120 s; 5 cycles at 95 °C for 60 s, 63 °C for 60 s, and 72 °C for 60 s; 25 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 12 min. PCR products were inspected on 1.5% agarose gel.

Basic like-parentage assignment was performed using seven microsatellite markers developed for sturgeon species including Afu_19, Afu_68[51]; Aox_27, Aox_45[52]; Spl_107, Spl_163 and Spl_173[53]. Microsatellites were amplified according to protocol of Havelka, *et al.*[54]. Forward primers within each of the 7 primer sets possessed a 5′prime end tail (M13R). During PCR, a fluorescently labelled primer (M13R) was added to the standard amplification reaction. Touchdown PCR protocol was employed for thermal cycling: initial denaturation 95 °C for 3 min followed by 10 cycles of 95 °C for 45 s, 65 °C–1 °C per cycle for 45 s and 72 °C for 45 s, followed by 20 cycles of 95 °C for 45 s, 53 °C for 45 s and 72 °C for 45 s, and final extension 72 °C for 10 min. Microsatellite fragment analysis was performed on a 3130xl ABI Genetic Analyser (Applied Biosystems, TM) using a GeneScan LIZ 500 size standard (Applied Biosystems, TM), and genotypes were scored in GENEIOUS 6.1.8[55] using Microsatellite Plugin 1.4.

References

1. Wildt, D. E. Genetic resource banks for conserving wildlife species: Justification, examples and becoming organized on a global basis. *Anim. Reprod. Sci.* **28**, 247–257 (1992).
2. Wells, D. N., Misica, P. M., Tervit, H. R. & Vivanco, W. H. Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reprod. Fertil. Dev.* **10**, 369–378 (1998).
3. Lanza, R. P. *et al*. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* **2**, 79–90 (2000).
4. Solti, L., Crichton, E. G., Loskutoff, N. M. & Cseh, S. Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. *Theriogenology* **53**, 149–162 (2000).
5. Loi, P. *et al*. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat. Biotechnol.* **19**, 962–964 (2001).
6. Labbé, C., Robles, V. & Herraez, M. P. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In book: Advances in Aquaculture Hatchery Technology (eds Allan, G. & Burnell, G) 76–116 (Elsevier, 2013).
7. Srirattana, K. *et al*. Full-term development of gaur-bovine interspecies somatic cell nuclear transfer embryos: Effect of trichostatin A treatment. *Cell. Reprogram.* **14**, 248–257 (2012).
8. Gómez, M. C., Pope, C. E. & Dresser, B. L. Nuclear transfer in cats and its application. *Theriogenology* **66**, 72–81 (2006).
9. Wani, N. A., Vettical, B. S. & Hong, S. B. First cloned Bactrian camel (*Camelus bactrianus*) calf produced by interspecies somatic cell nuclear transfer: A step towards preserving the critically endangered wild Bactrian camels. *PLoS ONE* **12(5)**, e0177800 https://doi.org/10.1371/journal.pone.0177800 (2017).
10. Birstein, V. J., Bemis, W. E. & Waldman, J. R. The threatened status of acipenseriform species: a summary. *Environ. Biol. Fish.* **48**, 427–435 (1997).
11. International Union for Conservation of Nature (2017). The IUCN Red List of Threatened Species, Version 2017–2, www.iucnredlist.org (accessed on 14 September 2017).
12. Birstein, V. J. & Vasil'ev, V. P. Tetraploid-octoploid relationships and karyological evolution in the order Acipenseriformes (Pisces): karyotypes, nucleoli, and nucleolus-organizer regions in four acipenserid species. *Genetica* **73**, 3–12 (1987).
13. Fontana, F. *et al*. Karyotypic characterization of the great sturgeon, *Huso huso*, by multiple staining techniques and fluorescent *in situ* hybridization. *Mar. Biol.* **132**, 495–501 (1998).
14. Tagliavini, J. *et al*. Molecular cytogenetic analysis of the karyotype of the European Atlantic sturgeon. *Acipenser sturio. Heredity* **83**, 520–525 (1999).
15. Fontana, F., Lanfredi, M., Rossi, R., Bronzi, P. & Arlati, G. Karyotypic characterization of *Acipenser gueldenstaedti* with C-, AgNO3 and fluorescence banding techniques. *Ital. J. Zool.* **63**, 113–118 (1996).
16. Vasil'ev, V. P., Vasil'eva, E. D., Shedko, S. V. & Novomodny, G. V. Ploidy levels in the kaluga *Huso dauricus* and sakhalin sturgeon *Acipenser mikadoi* (Acipenseridae, Pisces). *Dokl. Biol. Sci.* **426**, 228–231 (2009).
17. Fontana, F. *et al*. Evidence of hexaploid karyotype in shortnose sturgeon. *Genome* **51**, 113–119 (2008).
18. Zhu, Z. Y. & Sun, Y. H. Embryonic and genetic manipulation in fish. *Cell Res.* **10**, 17–27 (2000).
19. Lee, K. Y., Huang, H., Ju, B., Yang, Z. & Lin, S. Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat. Biotechnol.* **20**, 795–799 (2002).
20. Ju, B. *et al*. Development and gene expression of nuclear transplants generated by transplantation of cultured cells nuclei into non-enucleated eggs in the medaka *Oryzias latipes*. *Dev. Growth Differ.* **45**, 167–174 (2003).
21. Kaftanovskaya, E., Motosugi, N., Kinoshita, M., Ozato, K. & Wakamatsu, Y. Ploidy mosaicism in well-developed nuclear transplants produced by transfer of adult somatic cell nuclei to non-enucleated eggs of medaka (*Oryzias latipes*). *Dev. Growth Differ.* **49**, 691–698 (2007).
22. Wakamatsu, Y. Novel method for the nuclear transfer of adult somatic cells in medaka fish (*Oryzias latipes*): use of diploidized eggs as recipients. *Dev. Growth Differ.* **50**, 427–436 (2008).
23. Siripattarapravat, K., Pinmee, B., Venta, P. J., Chang, C. C. & Cibelli, J. B. Somatic cell nuclear transfer in zebrafish. *Nat. Methods* **6**, 733–735 (2009a).
24. Luo, D. J., Hu, W., Chen, S. P. & Zhu, Z. Y. Critical developmental stages for the efficiency of somatic cell nuclear transfer in zebrafish. *Int. J. Biol. Sci.* **7**, 476–486 (2011).
25. Liu, T. M. *et al*. Factors affecting the efficiency of somatic cell nuclear transplantation in the fish embryo. *J. Exp. Zool.* **293**, 719–725 (2002).
26. Akimenko, M. A., Mari-Beffa, M., Becerra, J. & Geraudie, J. Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev. Dynam.* **226**, 190–201 (2003).
27. Chenais, N., Depincé, A., Le Bail, P. Y. & Labbé, C. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of finfish genetic resources. *Aquac. Int.* **22**, 63–76 (2014).
28. Le Bail, P. Y. *et al*. Optimization of somatic cell injection in the perspective of nuclear transfer in goldfish. *BMC Dev. Biol.* **10**, 64 (2010).
29. Meissner, A. & Jaenisch, R. Mammalian nuclear transfer. *Dev. Dynam.* **235**, 2460–2469 (2006).
30. Hochleithner, M. & Gessner, J. The Sturgeons and Paddlefishes (Acipenseriformes) of the World - Biology and Aquaculture. 1–248 (AquaTech Publications, 2012).
31. Siripattarapravat, K., Busta, A., Steibel, J. P. & Cibelli, J. Characterization and *in vitro* control of MPF activity in zebrafish eggs. *Zebrafish* **6**, 97–105 (2009b).
32. Bubenshchikova, E. *et al*. Generation of fertile and diploid fish, medaka (*Oryzias latipes*), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. *Cloning Stem Cells* **7**, 255–264 (2005).
33. Gasaryan, K. G., Hung, N. M., Neyfahk, A. A. & Ivanenkov, V. V. Nuclear transplantation in teleost *Misgurnus fossilis* L. *Nature* **280**, 585–587 (1979).
34. Tung, T. C. *et al*. Nuclear transplantation in fishes. *Sci. Sin.* **14**, 1244–1245 (1963).
35. Gibbs, P. D. L., Peek, A. & Thorgaard, G. An *in vivo* screen for the luciferase transgene in zebrafish. *Mol. Mar. Biol. Biotechnol.* **3**, 307–316 (1994).
36. Lee, K. W., Webb, S. E. & Miller, A. L. A wave of free cytosolic calcium traverses zebrafish eggs on activation. *BMC Dev. Biol.* **214**, 168–180 (1999).
37. Depincé, A., Marandel, L., Goardon, L., Le Bail, P. Y. & Labbé, C. Trout coelomic fluid suitability as Goldfish oocyte extender can be determined by a simple turbidity test. *Theriogenology* **75**, 1755–1761 (2011).
38. Siripattarapravat, K. *et al*. The influence of donor nucleus source on the outcome of zebrafish somatic cell nuclear transfer. *Int. J. Dev. Biol.* **54**, 1679–1683 (2010).
39. Ikegami, R., Rivera-Bennetts, A. K., Brooker, D. L. & Yager, T. D. Effect of inhibitors of DNA replication on early zebrafish embryos: evidence for coordinate activation of multiple intrinsic cell-cycle checkpoints at the mid-blastula transition. *Zygote* **5**, 153–175 (1997).
40. Kane, D. A. & Kimmel, C. B. The zebrafish midblastula transition. *Development* **119**, 447–456 (1993).
41. Blelloch, R. *et al*. Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. *Stem Cells* **24**, 2007–2013 (2006).
42. Niemann, H., Tian, X. C., King, W. A. & Lee, R. S. F. Epigenetic reprogramming in embryonic and foetal development upon somatic cell nuclear transfer cloning. *Reproduction* **135**, 151–163 (2008).

43. Bubenshchikova, E. *et al*. Diploidized eggs reprogram adult somatic cell nuclei to pluripotency in nuclear transfer in medaka fish (*Oryzias latipes*). *Dev. Growth Differ.* **49**, 699–709 (2007).
44. Newport, J. & Kirschner, M. A Major Developmental Transition in Early *Xenopus* Embryos: I. Characterization and Timing of Cellular Changes at the Midblastula Stage. *Cell* **3**, 675–686 (1982).
45. Havelka, M., Kašpar, V., Hulak, M. & Flajšhans, M. Sturgeon genetics and cytogenetics: a review related to ploidy levels and interspecific hybridization. *Folia Zool.* **60**, 93–103 (2011).
46. Havelka, M., Fujimoto, T., Hagihara, S., Adachi, S. & Arai, K. Nuclear DNA markers for identification of Beluga and Sterlet sturgeons and their interspecific Bester hybrid. *Sci Rep.* **7**, 1694 (2017).
47. Cherr, G. N. & Clark, W. H. Jr. Fine structure of the envelope and micropyles in the eggs of the white sturgeon, *Acipenser transmontanus* Richardson. *Dev. Growth Differ.* **24**, 341–352 (1982).
48. Piferrer, F. *et al*. Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture* **239**, 125–156 (2009).
49. Yabe, T., Ge, X. & Pelegri, F. The zebrafish maternal-effect gene *cellular atoll* encodes the centriolar component *sas-6* and defects in its paternal function promote whole genome duplication. *Dev. Biol.* **312**, 44–60 (2007).
50. Sohrabnezhad, M., Kalbassi, M. R., Nazari, R. M. & Bahmani, M. Short-term storage of Persian sturgeon (*Acipenser persicus*) ova in artificial media and coelomic fluid. *J. Appl. Ichthyol.* **22**, 395–399 (2006).
51. May, B., Krueger, C. C. & Kincaid, H. L. Genetic variation at microsatellite loci in sturgeon: primer sequence homology in *Acipenser* and *Scaphirhynchus*. *Can. J. Fish Aquat. Sci* **54**, 1542–1547 (1997).
52. King, T. L., Lubinski, B. A. & Spidle, A. P. Microsatellite DNA variation in Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus*: and cross-species amplification in the Acipenseridae. *Conserv. Genet.* **2**, 103–119 (2001).
53. McQuown, E., Gall, G. A. E. & May, B. Characterization and inheritance of six microsatellite loci in lake sturgeon. *Trans. Am. Fish Soc.* **131**, 299–307 (2002).
54. Havelka, M., Hulak, M., Bailie, D. A., Prodöhl, P. A. & Flajšhans, M. Extensive Genome Duplication in Sturgeons: New Evidence from Microsatellite Data. *J. Appl. Ichthyol.* **29**, 704–708 (2013).
55. Kearse, M. *et al*. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649 (2012).

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Author Contributions

A.D. trained E.F. for nuclear transfer in fish and C.L. introduced E.F. to the scientific background on nuclear transfer in fish. T.S. designed the experiment with E.F., who conducted the experiments, analysed data and wrote the manuscript. M.H. performed and analysed the molecular data providing necessary suggestions on the text. M.P. performed sampling and managed the laboratory and with V.I. performed the ploidy analysis. All authors contributed to the preparation of the manuscript and approved the final version.

Additional Information

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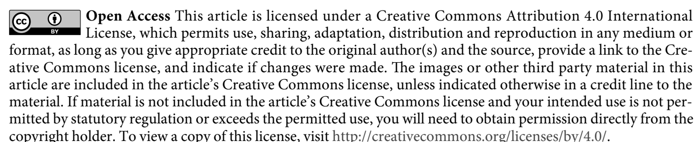

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Supplementary information

Video S1. Frontal view of NT-fish. Swelled heart is beeping at 5 days post activation.

Access the supplementary video S1 in the following address: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-018-24376-1/MediaObjects/41598_2018_24376_MOESM2_ESM.mov

Video S2. Lateral view of NT-fish. Swelled heart is beeping at 5 days post activation.

Access the supplementary video S2 in the following address: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-018-24376-1/MediaObjects/41598_2018_24376_MOESM3_ESM.mov

Video S3. 27 days post activation. The NT-larva is being fed with tubifex sp.

Access the supplementary video S3 in the following address: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-018-24376-1/MediaObjects/41598_2018_24376_MOESM4_ESM.mov

CHAPTER 3

A NEWLY DEVELOPED CLONING TECHNIQUE IN STURGEONS; AN IMPORTANT STEP TOWARDS RECOVERING ENDANGERED SPECIES

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A newly developed cloning technique in sturgeons; an important step towards recovering endangered species

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Several steps of sturgeon somatic cell nuclear transfer (SCNT) have been recently established, but improvements are needed to make it a feasible tool to preserve the natural populations of this group of endangered species. The donor cell position inside the recipient egg seems to be crucial for its reprogramming; therefore by injecting multiple donor somatic cells instead of a single cell with a single manipulation, we increased the potential for embryo development. Using the Russian sturgeon *Acipenser gueldenstaedtii* as a multiple cell donor and sterlet *Acipenser ruthenus* as the non-enucleated egg recipient, we obtained higher proportion of eggs developing into embryos than previously reported with single-SCNT. Molecular data showed the production of a specimen (0.8%) contained only the donor genome with no contribution from the recipient, while two specimens (1.6%) showed both recipient and donor genome. These findings are the first report of donor DNA integration into a sturgeon embryo after interspecific cloning. In all, we provide evidence that cloning with the multiple donor somatic cells can be feasible in the future. Despite the fact that the sturgeon cloning faces limitations, to date it is the most promising technique for their preservation.

The Acipenseridae is an ancient family that faces internal and external threats including the loss of species genetic integrity through frequent interspecific hybridization[1], habitat degradation, and overfishing for their roe processed into caviar[2]. Dramatic decrease in sturgeon populations attracted attention of the International Union for Conservation of Nature (IUCN) that categorized them as the most critically endangered, more than any other group of species. Indeed, all 27 sturgeon species are on the IUCN Red List of threaten species with 17 categorized as critically endangered and four considered to be extinct[3].

Somatic cell nuclear transfer (SCNT) is an animal cloning technique that can aid conservation of species on the verge of extinction[4–11]. A single somatic cell from an endangered species can be reprogrammed to totipotency when placed in a favorable cytoplasmic environment of an easily reared species, producing an organism containing exclusively donor genomic material. Despite the demonstrated advantages of the method, SCNT is a challenging multi-step technique with low success even in model fish species[12–17]. The first crucial steps in sturgeon SCNT have been recently established and resulted in early embryogenesis of the nuclear transplants (NTs)[11]. However, utilizing a single fin cell harvested from an albino sterlet, *Acipenser ruthenus* and Russian sturgeon, *Acipenser gueldenstaedtii* transplanted into non-enucleated eggs from sterlet achieved low development rate of reconstructed NTs, of 18.1% and 12%, respectively[11].

The current study aims to improve the sturgeon SCNT technique by increasing the number of developing NTs that would be surrogate for gamete production. Introduction the donor genome into the recipient egg, with the long-term goal that the germline will produce the desired gametes. Results of studies in goldfish *Carassius auratus* have suggested that the cell injection position and depth inside the recipient egg are critical for donor cell reprogramming[18]. A single fin cell could be easily found in a non-favorable position inside the larger recipient egg. Egg

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Fin-cells donor species	Fin-cells/egg	Number of injected eggs	Blastula (%)	Gastrula-Blastopore formation (%)
Russian sturgeon	~330 ± 12	22	13 (59.1)	1 (4.5)
	~55 ± 3	34	5 (14.7)	0 (0)
Beluga	~240 ± 18	74	38 (51.4)	4 (5.4)
	~40 ± 2	56	18 (32.1)	1 (1.8)

Table 1. Comparison of mNTs developmental rate after different number of fin cells from Russian sturgeon or beluga were injected into sterlet eggs.

size varies in sturgeon species. Eggs of sterlet are smaller than those of Russian sturgeon and beluga, *Huso huso* (diameter 1.8–2.8 mm, 2.8–3.8 mm and 3.3–4.5 mm, respectively)[19]. Although sterlet eggs are the smallest among sturgeons[19], they are considerably larger than a single fin cell (~8 μm). No technique is currently available that will place the donor cell in the specific area of the recipient egg most conducive to its reprogramming. Therefore, SCNT is basically a blind technique, particularly challenging when using large recipient eggs as in sturgeon.

In the present study we injected multiple donor somatic cells instead of a single cell by a single manipulation, conducting multiple somatic cell nuclear transfer (mSCNT). The newly developed cloning technique increases the potential for the donor fin cell to be placed in the most favorable position inside the recipient sterlet egg and reprogrammed. We used sturgeon species most commonly exploited for caviar, the Russian sturgeon and the beluga[19], as donor species. Females and males Russian sturgeon are reproductively mature at 10–16 and 8–13 years, respectively[20], and beluga at 15–18 and 10–15 years, respectively[21]. These species are categorized as critically endangered[3], and therefore, fin tissue is an excellent source of donor genomic material, as the harvesting does not cause irreparable damage to the fish[10,22]. As a recipient cytoplasmic environment, non-enucleated and non-activated eggs of sterlet provide clear benefits[11]. Sterlet, classified as a vulnerable species[3], displays characteristics that make it a model species of the sturgeon family. It begins reproducing much earlier than the two mentioned species, 5–8 years in females and at 3–5 years in males[23]. Sterlet females spawn every 1–2 years[23] while Russian sturgeon and beluga spawn at 4–6[20] and 3–4 years[21] intervals, respectively. The use of sterlet as a recipient species and beluga as donor species has benefits for molecular genotyping using recently developed species-specific primers allowing routine identification of the NTs origin[24].

The current study improves the state of the art of interspecific SCNT for multiplying endangered animals. We transplanted multiple fin cells of albino sterlet, Russian sturgeon, or beluga into non-enucleated and non-activated sterlet eggs. We compared two numbers of fin cells in their ability to trigger embryonic development of the multiple nuclear transplants (mNTs). We assessed the donor cell fate in the developing mNTs and monitored embryo development. Finally, we assessed the donor cell genomic contribution to the developing transplants using molecular markers. The results of SCNT reported here represent a baseline that will contribute to progress in cloning Russian sturgeon and beluga for conservation purposes. Most importantly, the current research provides evidence that large critically endangered rare animals can be generated via the powerful interspecific SCNT technique.

Results

Importance of shallow donor cell injection in the animal pole of recipient egg. To evaluate the effect of deep donor cell injection in the central region of the recipient egg, we monitored development resulting from intraspecific and interspecific single-SCNT. No, initial cleavage was shown in the transplants and therefore shallow injection in the animal pole of the recipient egg was performed in the subsequent experiments.

Effect of the quantity of transferred donor cells in mNTs development. To evaluate mNT embryo development with respect to the number of transplanted fin cells, we monitored development resulting from two quantities of fin cells from Russian sturgeon (~330 ± 12 and ~55 ± 3/egg) and from beluga (~240 ± 18 and ~40 ± 2/egg) transplanted into sterlet eggs (Table 1). A significantly greater number of developing mNTs was observed with the higher number of injected cells compared to the lower quantity: 4-fold in Russian sturgeon mNTs ($P < 0.001$) and 1.6-fold in beluga mNTs, ($P < 0.05$) (Supplementary Fig. 1). The rate of mNTs development decreased in successive stages, with the number of both Russian sturgeon and beluga mNTs reaching gastrula lower compared to blastula (Table 1, Supplemetary Fig. 1). Considering these results, in subsequent experiments, we used the higher tested number of fin cells for mSCNT.

The fate of fin cells before and after microinjection. We visualized *in vivo* the fate of the fin cells immediately after dissociation as well as within the developing mNT. Two dyes were visible after the freshly dissociated fin cells from Russian sturgeon: the cells with intact or altered plasma membranes (red stain hiding the nucleus staining), as well as the released nuclei without plasma membrane staining (blue stain) (Fig. 1a). Inside the 8-cell stage developing Russian sturgeon mNT at six hours post-activation (hpa), due to many donor fin cells injected into a single area inside the egg, the released nuclei (blue stain) and the fin cell plasma membrane (red stain) are overlapping, resulting in a violet color. The violet color indicates the existence of blue stained released nuclei that overlap with red stained fin cell plasma membranes (intact or striped off) (Fig. 1b). We cannot exclude the possibility that some fin cell plasma membranes inside the sterlet egg collapsed, increasing the number of blue cells observed. After smashing the 8-cell stage Russian sturgeon mNT, both nuclei and plasma membranes were observed, indicating that plasma membranes of donor cells are not digested after injecting into the cytoplasm of oocyte, unlike the previous result reported in goldfish cloning[18] (Fig. 1c). The materials that appeared only in a bright filter image are probably debris from the smashed mNT (Fig. 1c).

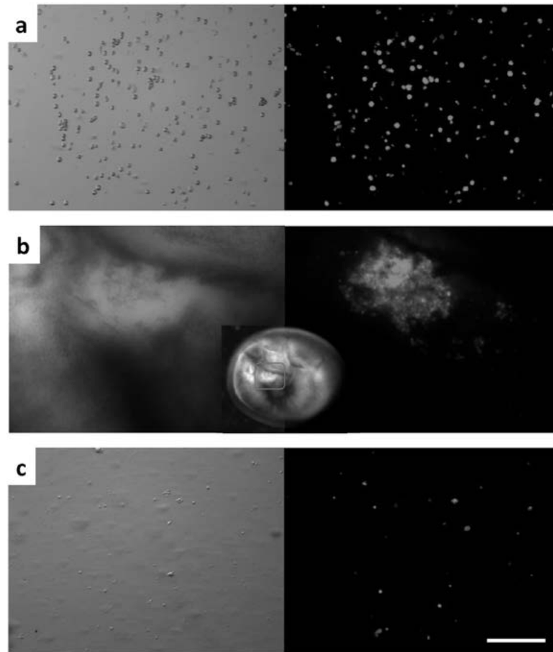

Figure 1. *In vivo* fluorescence analysis of fresh dissociated fin cells from Russian sturgeon. The left column represents bright field images while the right column shows the merging of UV and DsRED images. (**a**) Freshly dissociated fin cells before transplantation. The red color indicates the fin cell plasma membrane while the blue color indicates the released fin cell nuclei in cells with altered or striped off plasma membranes. (**b**) Visualization of the multiple fin cells inside the 8-cell stage developing Russian sturgeon mNT (corresponding to 6 hpa). The red color indicates the fin cell plasma membrane (intact or striped off) and the violet color indicates existence of released nuclei (blue color) that overlapped with the fin cell plasma membrane (red color). (**c**) Nuclei appeared after smashing the 8-cell stage Russian sturgeon mNT. Scale bar corresponds to 200 μm.

Histology. The presence of a nucleus inside a single blastomere of early blastula Russian sturgeon mNTs (256-cell stage, corresponding to 11 hpa) and sterlet control at the same time post-activation showed that the formed blastomeres were the result of embryological cleavage and not a mechanical stress response to microinjection (Fig. 2).

Effectiveness of mSCNT technique and mNTs development. The albino sterlet mNTs that displayed early cleavage reached blastula (44.4%) and two embryos (11.1%) succesfully formed the blastopore in gastrula before ceasing development. After the Russian sturgeon mSCNT, 85 Russian sturgeon mNTs that displayed early cleavage reached blastula (66.4%), and seven formed the blastopore in the gastrula (5.5%) but did not develop further. Sixty-eight beluga mNTs that displayed initial cleavage reached blastula (52.3%), eight formed the blastopore (6.2%), and three (2.3%) reached 2/3 of epiboly in the gastrula. In conclusion, all mNTs that displayed initial cleavage fullfilled blastula and either ceased development or developed further. After *in vitro* fertilization, more than 90% of sterlet control embryos showed development (n = 320). No extender-solution-only injected negative control (n = 100), showed cleavage furrows, as no somatic cell was injected (Table 2).

Phenotype of resulted mNTs. All mNTs produced developed normally, following the pattern of the sterlet control group. Immediately prior to cessation of development, the majority of mNTs formed the dorsal blastopore lip in the gastrula, with the exception of three beluga mNTs that developed further, forming 2/3 epiboly of the gastrula (Fig. 3).

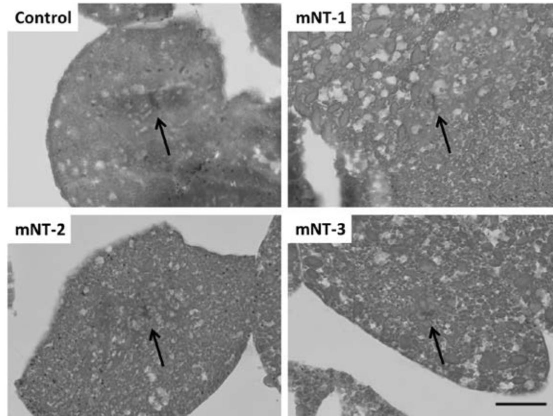

Figure 2. Histological sections of early blastula stage (256-cell) sterlet embryo (Control) and three Russian sturgeon mNTs (mNT-1, mNT-2 and mNT-3) at the same age. Black arrows indicate the location of the nuclei inside single blastomeres. Scale bar corresponds to 25 μm.

Experimental group	Total number of eggs	Blastula (%)	Gastrula (%)	
			Blastopore formation	2/3 Epiboly
Fertilized control	351	320 (91.2)	310 (88.3)	310 (88.3)
Extender-injected control	100	0 (0)	0 (0)	0 (0)
Albino sterlet mNTs	18	8 (44.4)	2 (11.1)	0 (0)
Russian sturgeon mNTs	128	85 (66.4)	5 (3.9)	0 (0)
Beluga mNTs	130	68 (52.3)	8 (6.2)	3 (2.3)

Table 2. Developmental rates of the reconstructed embryos (albino sterlet mNTs or Russian sturgeon mNTs or beluga mNTs) after transplantation of fin-cells originated from albino sterlet or Russian sturgeon or beluga, respectively, into sterlet eggs (~329 ± 2 or ~330 ± 12 or ~240 ± 18 fin-cells/egg, respectively). Sterlet control groups are presented at each developmental stage respectively to mNTs.

Two developing Russian sturgeon mNTs (2.4%) formed blastomeres with atypically large-volume cells at the 4-cell stage, corresponding to 5 hpa. However, at the late blastula corresponding to 14 hpa, they exhibited no phenotypic difference from the sterlet control group; both developed the blastopore in the gastrula and then ceased development. A single Russian sturgeon mNT exhibited a visible injury throughout its development, probably due to microinjection (Fig. 4; Supplementary Movie 1).

Use of molecular markers for mNTs identification. To evaluate the genetic origin of the mNTs, molecular analysis of Russian sturgeon and beluga mNTs was performed on the gastrula. The albino sterlet mNTs did not develop sufficiently for identification based on phenotype. All three beluga mNTs presented amplification of the sterlet 247 bp band using the *247_Arp + 247_uni* primer pair, while no amplification of the beluga 153 bp band with primer pair *153_HHp + 153_uni* was detected, demonstrating that they contained genome of the sterlet recipient only with no contribution of the donor beluga (Supplementary Fig. 2).

After interspecific mSCNT, the Russian sturgeon mNT-4 and the Russian sturgeon mNT-5 did not show any private allele of Russian sturgeon donor and they originated from recipient genome only (Table 3; Supplementary Fig. 3; Supplementary Table 1). The specimens Russian sturgeon mNT-6, Russian sturgeon mNT-7 and Russian sturgeon mNT-8 contained private alleles of Russian sturgeon donor at all informative loci (Supplementary Table 1). The specimens Russian sturgeon mNT-6 and Russian sturgeon mNT-7 showed concurrent occurrence of the recipient genome as evidenced by the presence of recipient's private alleles in the specimens' allele phenotypes and from amplification of 247 bp band by primer pair *247_ARp + 247_uni* (Table 3). On the contrary, the specimen Russian sturgeon mNT-8 did not possess any recipient private allele and displayed no amplification by primer pair *247_ARp + 247_uni* (Supplementary Fig. 3). It showed that this specimen contained only donor's genome with no contribution from the recipient (Table 3; Supplementary Fig. 3; Supplementary Table 1).

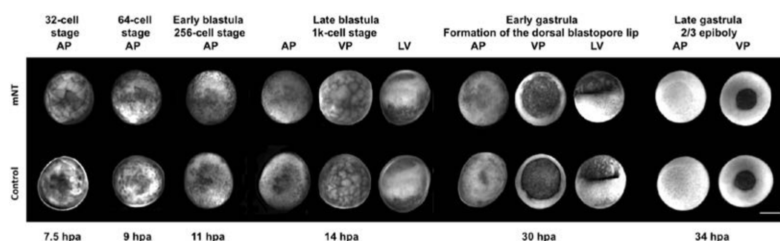

Figure 3. Comparison of the embryonic development between a beluga mNT and a sterlet control embryo. The embryonic stages, from early developmet (32-cell stage) until late gastrula (2/3 epiboly) are presented with the respective hours post activation (hpa) of the eggs. Animal pole (AP), vegetal pole (VP), lateral view (LV), scale bar corresponds to 1 mm.

Discussion

The main importance of our study lies in the optimization of the animal cloning technique in sturgeon. A major feature of interspecific mSCNT was the production of a gastrula stage embryo that contained only Russian sturgeon donor genome. This unique result after sturgeon SCNT is providing evidence for potential regeneration of populations of large animals with high commercial and ecological value by interspecific SCNT.

In our previous study on interspecific single-SCNT, we established several crucial steps specific to sturgeon biology, obtaining 12% early development of the NTs[11]. Although developed in a separate set of experiment that may complicate direct comparison, the current study using interspecific mSCNT produced higher percentage of early developing transplants (×5.5), even though the surviving embryos did not develop as far as in our former study[11]. We believe that mSCNT is a very promising strategy to increase the clone production because with this technique, we were able to obtain for the first time a gastrula stage mNT of only donor's origin and two gastrula stage mNTs bearing both the recipient and donor genome. Besides, mSCNT is more effective than single-SCNT since we are able to produce twice the amount of nuclear transplants in half the time of SCNT technique.

Our first hypothesis to explain the higher number of developing transplants using mSCNT is that the transplantation of multiple cells increases the likelihood of placement in the recipient egg optimal for development. A six-fold number of injected fin cells yielded 4- and 1.6-fold early developing Russian sturgeon and beluga mNTs, respectively.

A second hypothesis, suggested by *in vivo* fluorescence analysis where we investigated the fate of freshly dissociated fin cells, is that the mSCNT may have an advantage over single-SCNT due to injection of both intact fin cells and those with altered membranes. Alteration of the donor cell plasma membrane could help the injected exposed nucleus to activate easier the recipient egg. In mammalian SCNT, it is common practice to disrupt the plasma membrane of the somatic cell prior to transplantation[25–27], although in goldfish it has been shown that injection of the entire fin cell allows embryo development[18]. However, Le Bail *et al.*[18] reported that the fin cell plasma membrane was spontaneously disrupted seconds after transplantation, something not observed in the present work, *i.e.* red stained membranes were visible in 8-cell stage Russian sturgeon mNT (6 hpa). This may indicate that the sterlet ooplasm did not digest the donor plasma membrane. For that reason, we suggest that the hypothesis of the exposed nucleus can be valid in sturgeon cloning. It must be kept in mind that when intact somatic cells were injected, their cytoplasmic content was injected as well. If released from the cells after injection, this interphasic cytoplasm may have interfered with the metaphasic egg cytoplasm and have altered the embryonic clock upon egg activation and the onset of meiosis resumption. We have no clue about the extent of this phenomenon which may explain why some transplants did not develop. However, release of cytoplasm may not be thorough as indicated by the number of cells with their plasma membranes still observed at the 8-cell stage.

After mSCNT, all developing mNTs displayed development similar to the pattern of the sterlet control embryos. Sturgeon eggs may possess a defense mechanism to retain only a single fin cell and to absorb and eliminate other cells. To support that only one fin cell donor activates the recipient egg, we focused on the early development of the mNTs. In sturgeon experiments, both *in vivo*[28] and *in vitro*[29], eggs fertilized by multiple spermatozoa resulted in an abnormal number of blastomeres during early development. Iegorova *et al.*[29] showed that, generally, only one spermatozoon fuses with the egg nucleus and that the sturgeon eggs eliminate the remaining spermatozoa and exhibit a normal phenotype. However, occasionally the surplus spermatozoon/spermatozoa developed further as haploid cells resulting in formation of extra blastomeres[29]. In mSCNT experiments, if we consider fin cells to be analogous to spermatozoa, we can support the hypothesis that only one fin cell activated and triggered the development of the mNTs, because they were characterized by the normal number of blastomeres. However, validation of this hypothesis will require improvement of the mSCNT technique to generate hatched mNTs to assess genotype and ploidy.

We cannot conceal that during development, two Russian sturgeon mNTs exhibited atypically large blastomeres that coexisted with those of normal size. This may have been due to absence of mitotic events at this area or to slower division, either of which could lead to apoptosis, which would explain the development arrest. However, their cleavage division progressed similar to that of the sterlet control group, and the formed blastomeres were

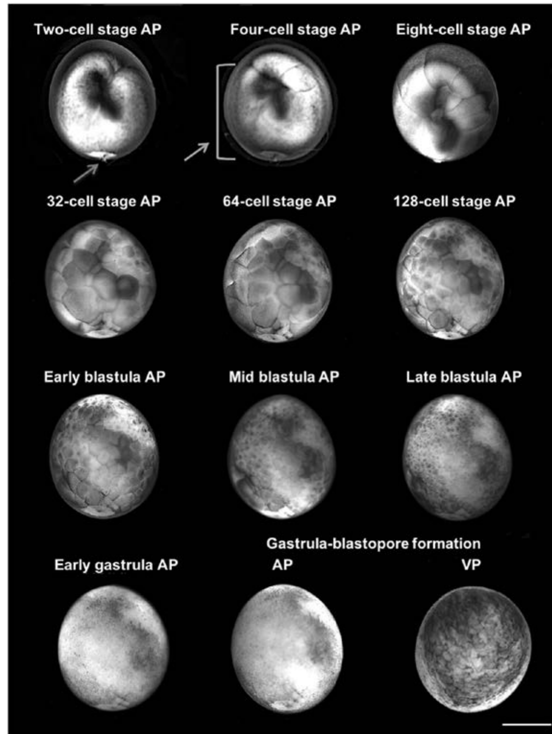

Figure 4. Development of a Russian sturgeon mNT that exhibit large blastomeres together with the normal size ones. The red arrow shows an injury position due to microinjection that remains throughout the development (gastrula-blastopore formation). The blue arrow is showing the area where the large blastomeres were created, mostly on the center and left side of the mNT. These blastomeres formed at the 4-cell developmental stage and were no longer visible in the late blastula stage. Animal pole (AP), vegetal pole (VP), scale bar corresponds to 1 mm.

Specimens after interspecific mSCNT	Marker																		
		AciG_35		*Afu_19*		*Afu_68*		*AfuG_54*		*AfuG_135*		*Aox_27*		*Aox_45*		*Spl_101*		*Spl_163*	
	247_AR	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R
mNT-4	+	−	+	−	+	−	+	−	+	N/A	N/A	−	+	−	+	N/A	N/A	−	+
mNT-5	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	N/A	N/A	−	+
mNT-6*	+	+	+	+	N/A	+	+	+	N/A	+	+	+	N/A	+	+	+	N/A	+	N/A
mNT-7*	+	+	+	+	N/A	+	+	+	+	+	+	+	+	+	+	+	N/A	+	N/A
mNT-8*	−	+	−	+	N/A	+	N/A	+	N/A	+	N/A	+	N/A	+	−	+	N/A	+	N/A

Table 3. Molecular analysis using microsatellites markers after interspecific mSCNT. The Russian sturgeon mNTs-6 and 7 contain alleles from both Russian sturgeon donor and sterlet recipient. The Russian sturgeon mNT-8 dispays only Russian sturgeon donor genome. Name of specimens is from mNT-4 to 8 as they are different from specimen mNT-1 to 3 in Fig. 2. D = donor; R = recipient; + = informative allele(s) present in allele phenotype of the sample; − = informative allele(s) not present in allele phenotype of the sample; N/A = not informative locus. *Specimens possessing all informative alleles from the donor.

normal in number; hence the only difference from the sterlet control group was the large blastomeres from 4-cell stage to late blastula. We cannot support the hypothesis that the size of the Russian sturgeon mNTs' blastomeres was a factor in the induction of apoptosis, since development was halted at the same stage as the other Russian sturgeon mNTs. In keeping with our hypothesis that a single fin cell activated development, the surface of the larger blastomeres may provide space for the non-expressed donor nucleus digestion by the recipient egg. This can be supported by the fact that, at blastula, the Russian sturgeon mNTs with large blastomeres exhibited no phenotypic difference from the other Russian sturgeon mNTs or those of the sterlet control group. It is interesting to emphasize that the sterlet eggs showed high resistance to stressful conditions. Despite the "rough" manipulation due to microinjection, and the stress of multiple fin cell injection, most mNTs that showed early development reached to the gastrula even when an injury from the microinjection position was visible. This affirms the suggestion of Ginsburg and Dettlaff[28] of the value of sturgeon species for experimental embryological studies.

Similar to our previous results with single-SCNT[11], development of both NTs and mNTs from the current study was limited to the gastrula. However, the proportion of blastula mNTs that gastrulated was generally lower than seen with NTs[11]. Thus, after interspecific mSCNT, the number of blastula Russian sturgeon mNTs that reached gastrulation decreased abruptly 17 times compared to 1.4 times in Russian NTs from single-SCNT[11]. Conversely, in albino sterlet mSCNT, the number of blastula albino sterlet mNTs that reached gastrulation was similar to this of albino NTs: 4 and 3.3 times[11], respectively. The number of blastula beluga mNTs that gastrulated decreased 8.4 times. Therefore, we can suggest that the critical stage in sturgeon cloning could be to reach the early gastrula, as all developing transplants that showed initial cleavage fulfilled the blastula. In actinopterygian SCNT, the critical stage for the NTs to overcome is the mid-blastula[16–18,30]. This unique characteristic within the actinopterygians may be attributed to embryonic genome activation and mitotic checkpoint triggering that take place in the late blastula as has been observed in Russian sturgeon[31] rather than in the mid-blastula as seen in the higher teleosts. It is reasonable to assume that this applies to all sturgeons. In amphibian SCNT, the critical stage seems to be the late blastula[32,33], as demonstrated by Dettlaff *et al.*[31] who noted the similarity in egg structure and cleavage pattern of acipenserids and amphibians. This similarity is also expressed in the process of gastrulation, the changes in the morphology of the embryo, morphogenetic movements, and the fate map of acipenserids being similar with those of anurans[34]. Therefore, SCNT might be more easily employed in sturgeon than in higher teleosts.

After the interspecific mSCNT, 40% of the gastrula stage Russian sturgeon mNTs contained both recipient and donor genome, the first donor DNA integration reported in the embryo with sturgeon SCNT. This can be explained by the use of non-enucleated eggs. In medaka[35–38] and in zebrafish cloning[39], after single blastula nuclei transplant into non-enucleated unfertilized eggs, the NTs that grew to the adult stage expressed genetic markers of both donor and recipient. Following Fatira *et al.*[11], we utilized non-enucleated and non-activated sterlet eggs, which, in teleost SCNT, has been shown to have a positive result in goldfish[18]. In our study, one of the five-gastrula stage Russian sturgeon mNTs possessed only Russian sturgeon donor alleles. Bubenshchikova *et al.*[40] were the first to hypothesize a yet to be determined mechanism responsible for excluding the recipient nucleus from the NTs. This is the first report of a Russian sturgeon, a critically endangered species, generated from interspecific SCNT. This exclusively donor-DNA-derived embryo is evidence of the potential of assisted reproduction technology to conserve threatened population by interspecific SCNT[25,41,42]. The limitation of our result is that the Russian sturgeon mNT ceased development in the embryonic phase. As already discussed in teleost[43], fin cells bear a specific differentiated profile based on epigenetic marks which may not be accurately reprogrammed during SCNT. It was shown for example that numerous genes are differentially expressed between embryos obtained after fertilization and after nuclear transfer[44]. It is likely that in the present experiment, none of the donor cells were efficiently reprogrammed. It cannot be excluded either that the somatic cytoplasm incorporated during mSCNT impaired epigenetic reprogramming of the injected nuclei, and that a preliminary reprogramming treatment as in Chenais *et al.*[43] will be required with our technique.

This Russian sturgeon mNT from donor origin only lost the recipient DNA. It has been recently proposed in teleost[45] that after SCNT, the egg DNA remains under the first cleavage grove and is scattered without replication in the blastomeres upon successive cleavage, or that it is extruded as a whole with the second polar body, because of an alteration of the meiotic furrow.

Two of the five Russian sturgeon mNTs and the three beluga mNTs displayed only the recipient sterlet genome. This is in accordance with our previous experiment using interspecific single SCNT, in which the obtained NT larva displayed only recipient sterlet genome as a result of unusual disruption in early embryogenesis[11]. Gynogenesis has been observed after fish SCNT when non-enucleated recipient eggs were used: zebrafish intraspecific embryonic cell nuclear transfer resulted in adult diploid NTs (1.6%) that originated only from the recipient[39]. In medaka SCNT, adult NTs (1.0%) exhibited only recipient-derived markers[40]. These authors proposed that the combination of successful diploidization of the recipient nuclei in medaka with no active mitosis in donor-derived nuclei resulted in the formation of parthenogenic individuals[40]. In our experiments we did not diploidized the sterlet recipient genome prior to transplantation, but we know that this already happened accidently in Fatira *et al.*[11]. It cannot be excluded that some alteration of the egg plasma membrane during SCNT prevented the correct extrusion of the second meiotic polar body, resulting in the maintenance of a diploid recipient genome. We strongly suggest that beluga mSCNT will show positive results, as acipenserids can hybridize, resulting in viable progeny[46,47]. Species that hybridize naturally are more likely to perform better in interspecific SCNT[48], since viable hybrid offspring indicates that nuclear-cytoplasmic compatibility exists between the two species[49].

The present study provides evidence that interspecific cloning can be used for reproduction of critically endangered large animals. Improvements of the method according to species biology are necessary to develop an efficient tool for conservation of wild populations.

Methods

Ethics. The transplantation experiments took place at the Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in České Budějovice, Czech Republic. All procedures were performed in accordance with national (reference number: 2293/2015-MZE-17214) and institutional guidelines on animal experimentation and care and approved by the Animal Research Committee of the University of South Bohemia in České Budějovice.

Fish and gamete collection. In mSCNT experiments a single non-sexually-mature 2–3 year old albino sterlet, two Russian sturgeon (3–4 year old), and six beluga (3–4 month old) were used as donor species, and five mature 5–6 year old sterlet females were used as recipient species. *In vitro* fertilization of a sterlet control group was conducted using sperm from five mature male sterlet (6–7 year old). In deep single-SCNT experiments a non-sexually-mature 2–3 year old albino sterlet, and a Russian sturgeon (1–2 year old) were used as donor species, and one mature 6–7 year old sterlet female was used as recipient species. To perform *in vitro* fertilization of the sterlet control group sperm collected from a mature 7–8 year old sterlet male. Ovulation and spermiation were hormonally induced, and eggs and sperm were collected as described by Fatira *et al.*[11].

Donor fin cell preparation. In deep single-SCNT experiments caudal fin tissue (~0.5 cm^2) from albino sterlet (single experiment) and Russian sturgeon (single experiment) was clipped using sterile scissors. In mSCNT experiments caudal fin tissue (~0.5 cm^2) from albino sterlet (single experiment) and Russian sturgeon (two experiments, each experiment reflects the different cell quantity injected) as well as fin tissue from beluga (two experiments, each experiment reflects the different cell quantity injected) was clipped using sterile scissors. The fin fragments were dissociated into single cells according to Fatira *et al.*[11] 2–3 h before the mSCNT experiment. In mSCNT experiments the pellet containing fin cells from albino sterlet was suspended in 25 µl phosphate buffered saline (PBS), while the pellets containing Russian sturgeon or beluga fin cells were suspended in either 25 µl or 150 µl PBS. Cell viability and concentration were assessed using a hemocytometer (Bright-Line™ Hemacytometer; Hausser Scientific) after Trypan blue staining (SIGMA-ALDRICH®). The cells were kept at 4 °C until use.

Nuclear transfer. The recipient sterlet eggs were washed three times with Persian sturgeon artificial coelomic fluid (PSACF)[50] as described by Fatira *et al.*[11], and placed in a 6 cm^2 Petri dish filled with PSACF at 15 °C. Transplantation was conducted using a hydraulic injector (Cell-Tram Oil; Eppendorf, Germany) connected to a micromanipulator (MO-152; Narishige, Japan) under a stereomicroscope (Leica M165 FC.) according to Le Bail *et al.*[18]. To test if there is a favorable position in the large egg of sturgeon for donor nucleus, we performed deep single-SCNT, in which a single donor cell was transplanted into recipient oocytes at the depth of about 1 mm from the animal pole. In deep single-SCNT experiments we followed the transplantation procedure as described in Fatira *et al.*[11]. In mSCNT experiments each egg was held with a 0.7–0.8 mm glass capillary holder so that the animal pole faced the pulled-glass microcapillary needle (inner diameter: 25–28 µm) filled with 5 µl of fin cell suspension. A small quantity of fin cell suspension (~0.2 µl) was transferred into each sterlet egg (for albino sterlet ~329 ± 2 cells/egg, n = 18, for Russian sturgeon ~330 ± 12, n = 128 or ~55 ± 3 cells/egg, n = 34 and for beluga ~240 ± 18, n = 130 or ~40 ± 2 cells/egg, n = 56). After all mSCNT experiments (5 experiments), the albino sterlet mNTs (n = 18), the Russian sturgeon mNTs (n = 162) and the beluga mNTs (n = 186) were incubated in the PSACF (15 °C) for 30–40 min and then activated with filtrated water. Simultaneously, fertilization of the control group with freshly stripped sterlet sperm (5 experiments for mSCNT with 90–93% fertilization rate and a single experiment for deep single-SCNT with 95.7% fertilization rate) was performed to produce control sterlet embryos (n = 351 for mSCNT, n = 187 for deep single-SCNT). We used sterlet eggs with PSACF only (n = 100) as a negative control, activated in filtered water after 30–40 min incubation.

Treatment and culture of the embryos. Immediately after activation of all transplants and fertilization of the sterlet control group, all embryos were treated with 0.01% tannic acid (SIGMA-ALDRICH®) alternating with filtrated water for 10 min to remove the egg surface stickiness. Development was observed at the two-cell stage, corresponding to 3–4 hpa. At 10 hpa, forceps were used to remove the outer layers of chorion for better observation of the development. Developing embryos were placed in 0.01% penicillin and 0.01% streptomycin in filtered water at 15 °C for three days. All embryos were held at the ambient photoperiod at water temperature of 15 °C.

Statistical analysis. We used R software (v. 3.5.1) to compare the number of Russian sturgeon and beluga mNTs generated with injection of two numbers of fin cells. Non-developing mNTs and those developing to blastula and gastrula at each donor cell number were compared using Fisher's exact test. $P < 0.05$ was considered to be significant.

***In vivo* fluorescence observation of fin cells fate.** Prior to interspecific mSCNT, we labeled freshly dissociated fin cells from a Russian sturgeon with Hoechst 33342 (SIGMA-ALDRICH®) and PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (SIGMA-ALDRICH®) according to the manufacturer's instructions. After washing the stained cells to prevent injection of the dyes, we injected cells into sterlet recipient eggs (n = 2). We monitored the developing embryos and, at the 8 cell-stage we smashed the developing Russian mNTs with a coverslip. The released nuclei were identified with Hoechst 33342, and donor cell plasma membrane was detected with the PKH26. Stained cells were observed under fluorescence microscopy (Leica M165 FC) with ultraviolet light and Red Fluorescent Protein (DsRED) filter linked to the illuminator (Leica Kubler CODIX) to enhance the fluorescence. The merged images were processed with ImageJ software v.1.47.

Histology. Sturgeon embryos were embedded in plastic to maintain their lipoid-tissue structure. Three Russian mNTs and three sterlet controls at early blastula were placed in Bouin's fixative for 24 h and subsequently stored in 80% ethanol (EtOH), gradually replaced by 100% EtOH. Thereafter, the embryos were infiltrated with Technovit 7100 in a shaker as follows: 25% Technovit 7100 in EtOH for 12 h, 50% Technovit 7100 in EtOH for 12 h, 75% Technovit 7100 in EtOH for 12 h, 100% Technovit for 24 h (last step repeated twice). After the addition of the Technovit 7100 Hardener I and II, the embryos were placed in a mold for 24 h at −30 °C and subsequently polymerized in an incubator for 24 h at 60 °C. Samples were cut into 4 µm sections using Leica RM2235 and stained with hematoxylin and eosin. Observation of nucleus (purple dot) inside a single blastomere (pink surface) in Russian sturgeon mNTs showed that the formation of blastomeres is the result of development and not due to a stress-response caused by microinjection.

Molecular genotyping. Genomic DNA was extracted from caudal fin tissue of two Russian sturgeons fin donors, two sterlet egg recipients, one 3–4-month-old beluga (not fin donor) along with five Russian mNTs and three beluga mNTs at gastrula stage using GenElute Mammalian Genomic DNA Miniprep Kit (SIGMA-ALDRICH®) according to the manufacturer's instructions. The presence of the donor genome in the three beluga mNT embryos was investigated using beluga specific primer pair *153_HHp + 153_uni*[24] that amplifies 153 bp fragment of beluga DNA. Presence of the sterlet recipient genome in beluga mNTs was tested by sterlet specific primer pair *247_ARp + 247_uni*[24], which amplifies 247 bp fragment from sterlet DNA. All reactions were performed according to Havelka *et al.*[24] in two independent replicates. Because no nuclear DNA marker for identification of the Russian sturgeon genome is available, the presence of donor genome in the five Russian sturgeon mNTs was estimated by parentage-like assignment using nine microsatellite markers: *AciG_35*[51], *Afu_19*, *Afu_68*[52], *AfuG_54*, *AfuG_135*[53], *Aox_27*, *Aox_45*[54], *Spl_101*, *and Spl_163*[55]. Amplification and microsatellite fragment analysis were carried out according to the protocol described by Havelka *et al*.[56]. Genotypes were scored in GENEIOUS 8.1.9, using Microsatellite Plugin 1.4.4. The complexity of the duplicated sturgeon genome and the state of current microsatellite genotyping make it impossible to reliably determine allele dosage behind a specific peak. Hence, peak pattern was treated as dominant data and interpreted as allele phenotype[57]. Alleles that the Russian sturgeon donor did not share with the sterlet recipient (private alleles) were identified and tracked in allele phenotypes of Russian sturgeon mNTs. Together with microsatellite genotyping, sterlet specific primer pair *247_ARp + 247_AR* was used to confirm presence of the sterlet recipient genome in Russian sturgeon mNTs as described above.

References

1. Ludwig, A., Makowiecki, D. & Benecke, N. Further evidence of trans-Atlantic colonization of Western Europe by American Atlantic sturgeons. *Archaeofauna* **18**, 185–192 (2009).
2. Birstein, V. J., Bemis, W. E. & Waldman, J. R. The threatened status of acipenseriform species: a summary. *Environ. Biol. Fishes* **48**, 427–435 (1997).
3. IUCN. *The IUCN Red List of Threatened Species. Version 2018-1*, http://www.iucnredlist.org (Downloaded on 05 July 2018).
4. Wildt, D. E. Genetic resource banks for conserving wildlife species: justification, examples and becoming organized on a global basis. *Anim. Reprod. Sci.* **28**, 247–257 (1992).
5. Lanza, R. P. *et al*. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* **2**, 79–90 (2000).
6. Solti, L., Crichton, E. G., Loskutoff, N. M. & Cseh, S. Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. *Theriogenology* **53**, 149–162 (2000).
7. Li, Y. *et al*. Cloned endangered species takin (*Budorcas taxicolor*) by inter-species nuclear transfer and comparison of the blastocyst development with yak (*Bos grunniens*) and bovine. *Mol. Reprod. Dev.* **73**, 189–195 (2006).
8. Oh, H. J. *et al*. Cloning endangered gray wolves (*Canis lupus*) from somatic cells collected postmortem. *Theriogenology* **70**, 638–647 (2008).
9. Gómez, M. C. *et al*. Cloning endangered felids using heterospecific donor oocytes and interspecies embryo transfer. *Reprod. Fertil. Dev.* **21**, 76–82 (2009).
10. Labbé, C., Robles, V. & Herraez, M. P. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. 76–116. In: Advances in aquaculture hatchery technology (Elsevier, 2013).
11. Fatira, E. *et al*. Application of interspecific Somatic Cell Nuclear Transfer (iSCNT) in sturgeons and an unexpectedly produced gynogenetic sterlet with homozygous quadruple haploid. *Sci. Rep.* **8**, 5997 (2018).
12. Lee, K. Y., Huang, H., Ju, B., Yang, Z. & Lin, S. Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat. Biotechnol.* **20**, 795–799 (2002).
13. Ju, B. *et al*. Development and gene expression of nuclear transplants generated by transplantation of cultured cells nuclei into non-enucleated eggs in the medaka *Oryzias latipes*. *Develop. Growth Differ.* **45**, 167–174 (2003).
14. Kaftanovskaya, E., Motosugi, N., Kinoshita, M., Ozato, K. & Wakamatsu, Y. Ploidy mosaicism in well-developed nuclear transplants produced by transfer of adult somatic cell nuclei to nonenucleated eggs of medaka (*Oryzias latipes*). *Develop. Growth Differ.* **49**, 691–698 (2007).
15. Wakamatsu, Y. Novel method for the nuclear transfer of adult somatic cells in medaka fish (*Oryzias latipes*): Use of diploidized eggs as recipients. *Develop. Growth Differ.* **50**, 427–436 (2008).
16. Siripattarapravat, K., Pinmee, B., Venta, P. J., Chang, C. C. & Cibelli, J. B. Somatic cell nuclear transfer in zebrafish. *Nat. Methods* **6**, 733–735 (2009).
17. Luo, D. J., Hu, W., Chen, S. P. & Zhu, Z. Y. Critical developmental stages for the efficiency of somatic cell nuclear transfer in zebrafish. *Int. J. Biol. Sci.* **7**, 476–486 (2011).
18. Le Bail, P. Y. *et al*. Optimization of somatic cell injection in the perspective of nuclear transfer in goldfish. *BMC Dev. Biol.* **10**, 64 (2010).
19. Hochleithner, M. & Gessner, J. The Sturgeons and Paddlefishes (*Acipenseriformes*) of the World – Biology and Aquaculture. 1–248 (AquaTech Publications, 3rd edition, Kitzbühel, 2012).
20. Gesner, J., Freyhof, J. & Kottelat, M. *Acipenser gueldenstaedtii*. The IUCN Red List of Threatened Species: e.T232A13042340, https://doi.org/10.2305/IUCN.UK.2010-1.RLTS.T232A13042340.en (**2010a**).
21. Gesner, J., Chebanov, M. & Freyhof, J. *Huso huso*. The IUCN Red List of Threatened Species 2010: e.T10269A3187455, https://doi.org/10.2305/IUCN.UK.2010-1.RLTS.T10269A3187455.en (**2010b**).
22. Akimenko, M. A., Marí-Beffa, M., Becerra, J. & Géraudie, J. Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev. Dynam.* **226**, 190–201 (2003).

23. Gesner, J., Freyhof, J. & Kottelat, M. *Acipenser ruthenus*. The IUCN Red List of Threatened Species 2010: e.T227A13039007, https://doi.org/10.2305/IUCN.UK.2010-1.RLTS.T227A13039007.en (2010c).
24. Havelka, M., Fujimoto, T., Hagihara, S., Adachi, S. & Arai, K. Nuclear DNA markers for identification of Beluga and Sterlet sturgeons and their interspecific Bester hybrid. *Sci. Rep.* **7**, 1694 (2017).
25. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. S. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813 (1997).
26. Chesne, P. *et al*. Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat. Biotechnol.* **20**, 366–369 (2002).
27. Wani, N. A., Vettical, B. S. & Hong, S. B. First cloned Bactrian camel (*Camelus bactrianus*) calf produced by interspecies somatic cell nuclear transfer: A step towards preserving the critically endangered wild Bactrian camels. *PLoS One* **12**, e0177800 (2017).
28. Ginsburg, A. S. & Dettlaff, T. A. The Russian Sturgeon *Acipenser Güldenstädti*. Part I. Gametes And Early Development Up To Time Of Hatching. In: Dettlaff, T. A. & Vassetzky, S. G. (eds) Animal Species for Developmental Studies (Springer, Boston, MA, 1991).
29. Iegorova, V., Pšenička, M., Lebeda, I., Rodina, M. & Saito, T. Polyspermy produces viable haploid/diploid mosaics in sturgeon. *Biol. Reprod*. ioy092 (2018).
30. Siripattarapravat, K. *et al*. The influence of donor nucleus source on the outcome of zebrafish somatic cell nuclear transfer. *Int. J. Dev. Biol.* **54**, 1679–1683 (2010).
31. Dettlaff, T. A., Ginsburg, A. S. & Schmalhausen, O. I. Sturgeon Fishes. Developmental Biology and Aquaculture, ISBN: 3-540-54744-4 (Springer-Verlag Berlin Heidelberg New York, 1993).
32. Gurdon, J. B. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J. Embryol. Exp. Morphol.* **10**, 622–640 (1962).
33. Gurdon, J. B., Laskey, R. A. & Reeves, O. R. The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. *J. Embryol. Exp. Morphol.* **34**, 93–112 (1975).
34. Ballard, W. W. Morphogenetic movements and fate maps of vertebrates. *Am. Zool.* **21**, 391–399 (1981).
35. Niwa, K., Ladygina, T., Kinoshita, M., Ozato, K. & Wakamatsu, Y. Transplantation of blastula nuclei to non-enucleated eggs in the medaka, *Oryzias latipes*. *Dev. Growth. Dif.* **41**, 163–172 (1999).
36. Niwa, K., Kani, S., Kinoshita, M., Ozato, K. & Wakamatsu, Y. Expression of GFP in nuclear transplants generated by transplantation of embryonic cell nuclei from GFP-transgenic fish into nonenucleated eggs of medaka, *Oryzias latipes*. *Cloning* **2**, 23–34 (2000).
37. Wakamatsu, Y. & Ozato, K. *Cloning of Fish*. In: Principles of Cloning, 287–299 (2002).
38. Bubenshchikova, E. *et al*. Generation of fertile and diploid fish, medaka (*Oryzias latipes*), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. *Clon. Stem Cells* **7**, 255–264 (2005).
39. Hattori, M., Hashimoto, H., Bubenshchikova, E. & Wakamatsu, Y. Nuclear transfer of embryonic cell nuclei to non-enucleated eggs in zebrafish, *Danio rerio*. *Int. J. Biol. Sci.* **7**, 460–468 (2011).
40. Bubenshchikova, E. *et al*. Nuclear transplants from adult somatic cells generated by a novel method using diploidized eggs as recipients in medaka fish (*Oryzias latipes*). *Clon. Stem Cells* **10**, 443–452 (2008).
41. Kishigami, S., Wakayama, S., Hosoi, Y., Iritani, A. & Wakayama, T. Somatic cell nuclear transfer: Infinite reproduction of a unique diploid genome. *Exp. Cell Res.* **314**, 1945–1950 (2008).
42. Loi, P., Iuso, D., Czernik, M., Zacchini, F. & Ptak, G. Towards storage of cells and gametes in dry form. *Trends Biotechnol.* **31**, 688–695 (2013).
43. Chenais, N. *et al*. Nuclear import of Xenopus egg extract components into cultured cells for reprogramming purposes: a case study on goldfish fin cells. *Sci. Rep.* **9**, 2861 (2019).
44. Luo, D. *et al*. Identification of differentially expressed genes between cloned and zygote-developing zebrafish (*Danio rerio*) embryos at the dome stage using suppression subtractive hybridization. *Biol. Reprod.* **80**, 674–684 (2009).
45. Rouillon, C., Depincé, A., Chênais, N., Le Bail, P. Y. & Labbé, C. Somatic cell nuclear transfer in non-enucleated goldfish oocytes: understanding DNA fate during meiosis resumption and first cellular division. *Sci. Rep*. (submitted), bioRxiv 630194; doi: https://doi.org/10.1101/630194.
46. Gorshkova, G., Gorshkov, S., Gordin, H. & Knibb, W. Karyological study in hybrids of beluga, *Huso huso* (L.) and the Russian sturgeon *Acipenser gueldenstaedti* brandt. *Israel J. Aquacult.* **48**, 35–39 (1996).
47. Havelka, M. *et al*. Fertility of a spontaneous hexaploid male Siberian sturgeon, *Acipenser baerii*. *BMC Genet.* **15**, 5 (2014).
48. Lagutina, I. *et al*. Development, embryonic genome activity and mitochondrial characteristics of bovine-pig inter-family nuclear transfer embryos. *Reproduction* **140**, 273–85 (2010).
49. Mastromonaco, G. F., Favetta, L. A., Smith, L. C., Filion, F. & King, W. A. The influence of nuclear content on developmental competence of gaur x cattle hybrid *in vitro* fertilized and somatic cell nuclear transfer embryos. *Biol. Reprod.* **76**, 514–523 (2007).
50. Sohrabnezhad, M., Kalbassi, M. R., Nazari, R. M. & Bahmani, M. Short-term storage of Persian sturgeon (*Acipenser persicus*) ova in artificial media and coelomic fluid. *J. Appl. Ichthyol.* **22**, 395–399 (2006).
51. Börk, K. *et al*. Development of new microsatellite primers for green and white sturgeon. *Conserv. Genet.* **9**, 973–979 (2008).
52. May, B., Krueger, C. C. & Kincaid, H. L. Genetic variation at microsatellite loci in sturgeon: primer sequence homology in *Acipenser* and *Scaphirhynchus*. *Can. J. Fish. Aquat. Sci.* **54**, 1542–1547 (1997).
53. Welsh, A. B., Blumberg, M. & May, B. Identification of microsatellite loci in lake sturgeon, *Acipenser fulvescens*, and their variability in green sturgeon, *A. medirostris*. *Mol. Ecol. Notes* **3**, 47–55 (2003).
54. King, T. L., Lubinski, B. A., Spidle, A. P. & Microsatellite, D. N. A. variation in Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) and cross-species amplification in the Acipenseridae. *Conserv. Genet.* **2**, 103–119 (2001).
55. McQuown, E. C. *et al*. Microsatellite analysis of genetic variation in Sturgeon: new primer sequences for *Scaphirhynchus* and *Acipenser*. *Trans. Am. Fish. Soc.* **129**, 1380–1388 (2000).
56. Havelka, M., Hulák, M., Bailie, D. A., Prodöhl, P. A. & Flajšhans, M. Extensive genome duplications in sturgeons: new evidence from microsatellite data. *J. Appl. Ichthyol.* **29**, 704–708 (2013).
57. Rodzen, J. A., Famula, T. R. & May, B. Estimation of parentage and relatedness in the polyploid white sturgeon (*Acipenser transmontanus*) using a dominant marker approach for duplicated microsatellite loci. *Aquaculture* **232**, 165–182 (2004).

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Author Contributions

E.F. designed the experiments, conducted the experiments, analysed data and wrote the manuscript. T.S. performed the histology of the embryos, provided advice on the experimental design and on manuscript. M.H. performed and analysed the molecular data providing necessary suggestions on the text. M.P. performed sampling and managed the laboratory. M.H., C.L., A.D., M.P. and T.S. contributed to manuscript writing and approved the final version.

Additional Information

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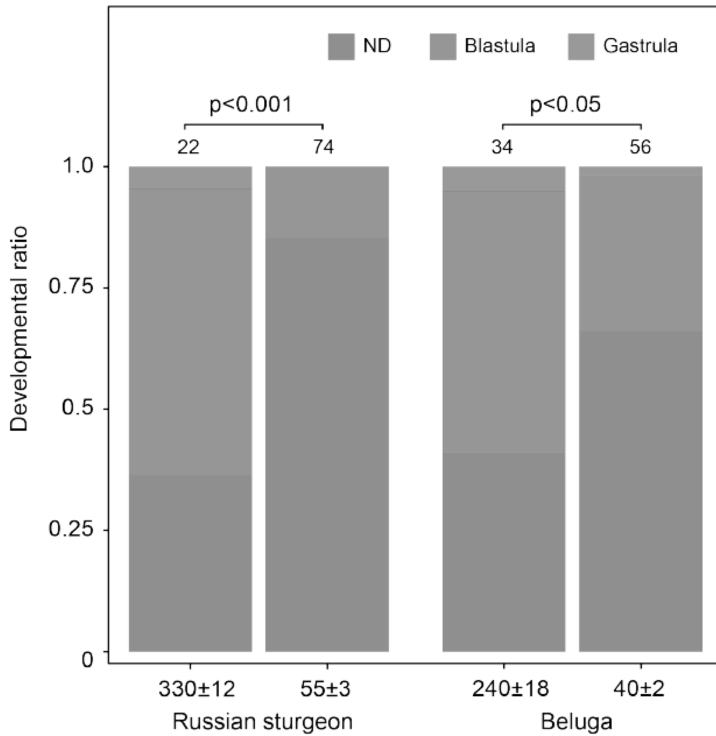
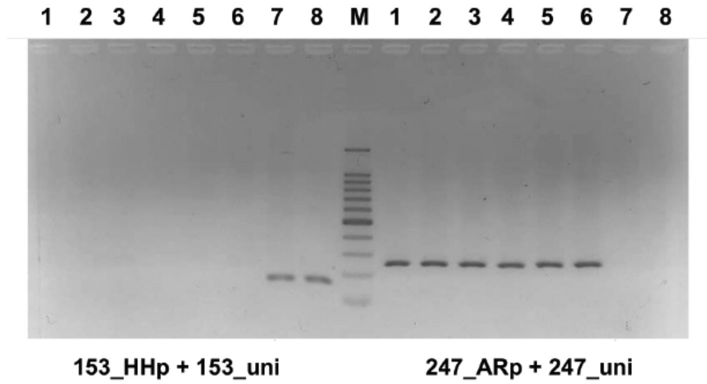
Supplementary information

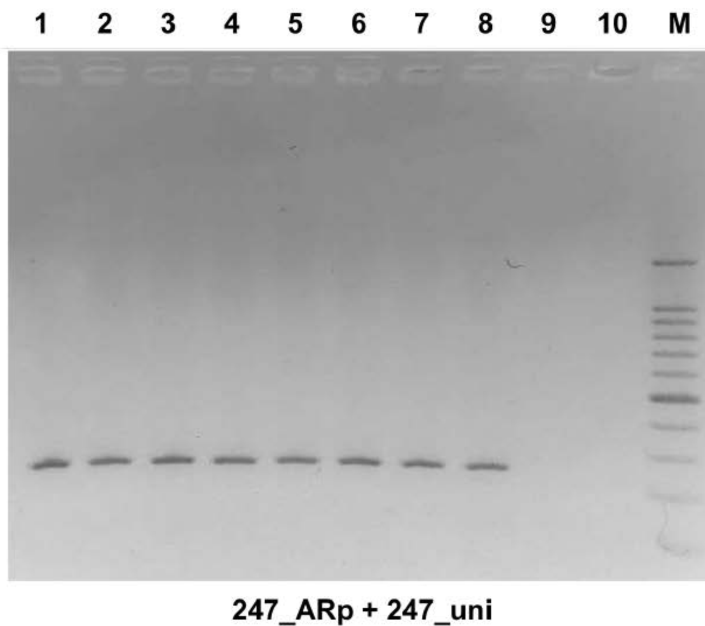


Supplementary Fig. 1. Higher number of embryos were activated and developed further than blastula stage when higher number of donor cells was injected. The number under the bars indicates the number of transplanted donor cells per egg. The numbers above the bars indicate the total number of embryos used for this analysis. ND= non-developing embryo; Blastula= embryos developed until blastula; Gastrula= embryos developed until gastrula.



Supplementary Fig. 2. Amplification by beluga specific primer pair *153_HHp + 153_uni* and sterlet specific primer pair *247_ARp + 247_uni*.

1-2 = beluga mNT-1; 3-4 = beluga mNT-2; 5-6 = beluga mNT-3; 7-8 beluga (fin-tissue control sample); M = 100 – 1500bp DNA ladder.



Supplementary Fig. 3. Amplification by sterlet specific primer pair *247_ARp* + *247_uni*. 1-2 = Russian sturgeon mNT-4; 3-4 = Russian sturgeon mNT-5; 5-6 = Russian sturgeon mNT-6; 7-8 = Russian sturgeon mNT-7; 9-10 = Russian sturgeon mNT-8; M = 100 – 1500bp DNA ladder.

Supplementary Movie 1. Development of a Russian sturgeon mNT that exhibit large blastomeres together with the normal size ones and a visible injury due to microinjection. The injury remains throughout the development (gastrula-blastopore formation), demonstrating the resilience of sturgeon embryos.

Supplementary Table 1. Genotyping results of Russian sturgeon multiple fin nuclear transfer specimens (Russian mNT-4 to 8) at 9 microsatellite loci.

AciG_35

ID	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
Russian Donor_I	268		276	280	284	286	288	292
Sterlet Recipient_I		272			284			292
Russian mNT-4		272						292
Russian mNT-5		272						292

ID	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9
Russian Donor_II	268		276	280	284	286	290	292	296
Sterlet Recipient_II		272			284				
Russian mNT-6	268	272	276	280	284	286	290	292	296
Russian mNT-7	268	272	276	280	284	286	290	292	296
Russian mNT-8	268		276	280	284	286	290	292	296

Afu_19

ID	Allele 1	Allele 2	Allele 3	Allele 4
Russian Donor_I	153	156		165
Sterlet Recipient_I		156	159	
Russian mNT-4		156		
Russian mNT-5			159	

ID	Allele 1	Allele 2	Allele 3	Allele 4
Russian Donor_II	153	156	159	165
Sterlet Recipient_II		156		
Russian mNT-6	153	156	159	165
Russian mNT-7	153	156	159	165
Russian mNT-8	153	156	159	165

Afu_68						
ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**	**Allele 5**	**Allele 6**
Russian Donor_I	150	154	162		210	234
Sterlet Recipient_I				206		
Russian mNT-4				206		
Russian mNT-5				206		

ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**	**Allele 5**	**Allele 6**	**Allele 7**
Russian Donor_II	150	154	162		210		266
Sterlet Recipient_II				198		238	
Russian mNT-6	150	154	162	198	210		266
Russian mNT-7	150	154	162		210	238	266
Russian mNT-8	150	154	162		210		266

AfuG_54						
ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**	**Allele 5**	**Allele 6**
Russian Donor_I			262	278	286	294
Sterlet Recipient_I	240	258				
Russian mNT-4	240	258				
Russian mNT-5	240	258				

ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**	**Allele 5**	**Allele 6**	**Allele 7**	**Allele 8**
Russian Donor_II		258		264	278	282	286	294
Sterlet Recipient_II	240		262					294
Russian mNT-6		258		264	278	282	286	294
Russian mNT-7	240	258	262	264	278	282	286	294
Russian mNT-8		258		264	278	282	286	294

AfuG_135				
ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**
Russian Donor_I	204		224	228
Sterlet Recipient_I	204	208		
Russian mNT-4	204			
Russian mNT-5		208		

ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**
Russian Donor_II	198	204		224
Sterlet Recipient_II			208	224
Russian mNT-6	198	204	208	224
Russian mNT-7	198	204	208	224
Russian mNT-8	198	204		224

Aox_27					
ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**	**Allele 5**
Russian Donor_I		152	156	160	164
Sterlet Recipient_I	136				
Russian mNT-4	136				
Russian mNT-5	136				

ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**	**Allele 5**
Russian Donor_II	132		152	160	164
Sterlet Recipient_II	132	136			
Russian mNT-6	132		152	160	164
Russian mNT-7	132	136	152	160	164
Russian mNT-8	132		152	160	164

Aox_45

ID	Allele 1	Allele 2	Allele 3	Allele 4
Russian Donor_I	136	145	161	172
Sterlet Recipient_I		145		
Russian mNT-4		145		
Russian mNT-5		145		

ID	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
Russian Donor_II	133			157	172
Sterlet Recipient_II		136	139		
Russian mNT-6	133		139	157	172
Russian mNT-7	133	136		157	172
Russian mNT-8	133			157	172

Spl_101

ID	Allele 1	Allele 2	Allele 3
Russian Donor_I	304	316	320
Sterlet Recipient_I		316	
Russian mNT-4		316	
Russian mNT-5		316	

ID	Allele 1	Allele 2	Allele 3	Allele 4
Russian Donor_II	304	316	320	
Sterlet Recipient_II			320	340
Russian mNT-6	304	316	320	
Russian mNT-7	304	316	320	
Russian mNT-8	304	316	320	

Spl_163				
ID	**Allele 1**	**Allele 2**	**Allele 3**	**Allele 4**
Russian Donor_I	196	204	220	228
Sterlet Recipient_I			220	
Russian mNT-4			220	
Russian mNT-5			220	

ID	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
Russian Donor_II	196	204		220	236
Sterlet Recipient_II			208	220	
Russian mNT-6	196	204	208	220	236
Russian mNT-7	196	204		220	236
Russian mNT-8	196	204		220	236

Supplementary Table 2. Intraspecific and interspecific deep single-SCNT and sterlet control group. Deep donor-cell injection into the centre of the recipient egg revealed no initial cleavage.

Experimental group	Total number of eggs	Initial embryonic cleavage (%)
Fertilized sterlet control	187	179 (95.7)
Intraspecific	120	0 (0.0)
Interspecific	130	0 (0.0)

CHAPTER 4

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

General discussion

The present Ph.D. study is introducing for the first time the animal cloning technique in critically endangered sturgeon species. Two distinctive parts separated into two scientific publications compose this study (Chapter 2 and 3). The first part (Chapter 2) established the basic steps of the nuclear transfer technique according to sturgeon's biology (Fatira et al., 2018), and the second part (Chapter 3) introduced a unique cloning technique adjusted for sturgeons as an improvement of the basic method (Fatira et al., 2019).

To start with, the first scientific study utilized both intraspecific and interspecific SCNT in which wild type sterlet was used as the egg recipient species and albino sterlet or the Russian sturgeon as the fin donor species (Fatira et al., 2018). Non-activated eggs were used because postponing the egg activation after nucleus injection was shown to increase the cloning success when compared to immediate activation (Le Bail et al., 2010). Probably donor nucleus exposure to the transcription factors deposited in a non-activated egg is favorable to reprogramming because non-activated eggs have high mitosis-promoting factor (MPF) activity (Siripattarapravat et al., 2009a) that is likely favorable to donor cell reprogramming. In addition, the resting time (30 min) that the study provided to the NTs before triggering meiosis resumption and first mitosis allowed some recovery from the mechanical disturbance induced by the nucleus injection.

Furthermore, in the present study have been used non-enucleated sterlet eggs according to Wakamatsu (2008), and Le Bail et al. (2010), because it was shown that the recipient egg genome was lost after nuclear transfer in medaka, *Oryzias latipes* (Bubenschikova et al., 2005) as well as in goldfish (Chênais et al., 2014). In addition to the recipient egg genome disappearance, skipping the enucleation step produced almost twice number of clones in the same experiment (Chênais et al., 2014). Also, when compared the use of enucleated and non-enucleated eggs after intraspecific ECNT from goldfish and bitterling fish, *Rhodeus sinensis* (Tung et al., 1963), and after SCNT from gynogenetic bighead carp into enucleated or non-enucleated gibel carp eggs (Liu et al., 2002), had shown no significant difference in the development of NTs embryos and hatchlings. In the present study sterlet egg enucleation was avoided mainly because of the risk of damage or losses of the maternal materials (i.e. proteins, mRNAs, mitochondria) that are necessary to support the developing embryo. Technical difficulty was also major limitation for using non-enucleated eggs in sturgeon because the eggs have a thick chorion, a very high internal pressure, and are extremely soft and breakable.

The first and perhaps the most crucial step for a successful SCNT was the use of a suitable extender solution that would maintain the ovulated eggs in metaphase II stage, that is the stage before the fertilization occurs and that would not damage the fin cells during the micromanipulation. In some fish species, coelomic fluid is used for SCNT, however, sturgeon coelomic fluid quality varies among females, it contains debris from the spawning that prevented the visualization of the donor cell and its viscosity is so high that impeded its aspiration into the microneedle. For these reasons, sturgeon coelomic fluid was not a good candidate to be used in the SCNT experiments. The artificial Persian sturgeon coelomic fluid (Sohrabnezhad et al., 2006) was chosen as an extender solution because the results demonstrated that the sterlet egg ability to be activated afterwards remained high during the experimental timeline. The results of the present study demonstrated that sturgeon eggs could remain inactivated even though they were incubated into a saline solution. Subsequently to SCNT, incubation of the NTs was held for 30 min before the activation, because it has been reported that incubation of eggs after transplantation for a certain period improves the developing rate of clones, probably by prompting the reprogramming of the donor nuclei (Siripattarapravat et al., 2009b; Le Bail et al., 2010). In addition, such a 30 min- incubation after sturgeon

SCNT was favorable as this time facilitated the micromanipulation of such a large egg (~20 nuclear transfers). In the series of research performed in the present Ph.D. thesis, it was not tested the correlation between the duration of incubation time after transplantation and the success rate of cloning. As part of future optimization of the sturgeon cloning technique, this correlation could be tested because the resting time after transplantation can be beneficial for donor cells to sustain reprogramming when their exposure time in the appropriate ooplasmic reprogramming factors is efficient.

Using the sterlet or the albino sterlet or Russian sturgeon as the donor fin-cell species and the sterlet as the recipient egg species, the present study could establish the basic steps of the nuclear transfer technique. After the iSCNT the study succeeded to obtain 12% early development of the NTs (i.e. 6.7% NTs development in comparison to 56% CNTRL development). Such a developing rate provided great hope for future success in sturgeon iSCNT since cloning is a very challenging multi-step technique with a low percentage of success even in model fish species. For example, cloning success in obtaining embryos after non-enucleation of goldfish egg is 17% (Le Bail et al., 2010), after enucleation of zebrafish egg the cloning success in obtaining adult individuals is 2% (Lee et al., 2002). In addition, cloning success in medaka after transplantation of freshly harvested fin-cells into diploidized eggs gave 2.7% (Bubenshchikova et al., 2007). Another possible explanation of NT's low success in the present study when used Russian sturgeon as a donor species is coming from Liu et al., (2002), which discussed that crossing species with different ploidy is an obstacle for embryonic development. This is because sterlet is functional diploid (Birstein and Vasil'ev 1987) and Russian sturgeon functional tetraploid (Fontana et al., 1996).

After the iSCNT technique between the Russian sturgeon and sterlet, six samples were molecularly analyzed by sterlet positive primer pair 247_AR + 247_uni and subsequently by sterlet negative primer pair 247_ARn + 247_uni as described in Havelka et al. (2017). In addition, basic parentage assignment was performed using seven microsatellite markers developed for sturgeon species including Afu_19, Afu_68 (May et al., 1997); Aox_27, Aox_45 (King et al., 2001); Spl_107, Spl_163, and Spl_173 (Mc Quown et al., 2002). Microsatellites were amplified according to the protocol of Havelka et al. (2013) and with the sterlet specific markers showed that the produced hatched larva obtained after iSCNT, contained only the egg recipient sterlet genome. In addition, the larva was homozygous in all loci that egg recipient was heterozygous and after ploidy analysis proved to be a quadruple haploid sterlet. As discussed in Chapter 2, this result was thought to be explained as a disturbance in early embryonic development after iSCNT (Chapter 2, Figure 7). However, the reason that occurred after iSCNT it is unknown, and it needs to be studied in the future.

In all, the Chapter 2 composes an important study because several steps of the SCNT technique were established (Fatira et al., 2018) that paved the way for the optimization of iSCNT in sturgeon (Fatira et al., 2019). Indeed, the second part of the present Ph.D. study focused on the improvement of the sturgeon cloning technique, by increasing the number of donor cells to be injected in a recipient egg (Chapter 3).

Results of studies in goldfish have suggested that the cell injection position and depth inside the recipient egg are critical for donor cell reprogramming (Le Bail et al., 2010). In Chapter 2 and 3, the microinjection performed in the animal pole of the sterlet eggs, were the multiple micropyles are located and that are the natural gates of the spermatozoa in normal fertilization process. When comes to SCNT technique, there is not a mechanism to place the donor cell in a favorable reprogramming position inside the recipient egg. In case of sterlet eggs although they are the smallest among sturgeons (diameter 1.8–2.8 mm) (Hochleithner and Gessner, 2012), are considerably larger than a single fin cell (~8 µm) and it is very easy for the donor cell to be placed in an non-favorable position for reprogramming. In Chapter 3,

microinjection of multiple donor fin cells instead of a single fin cell was performed by a single manipulation in sterlet eggs, in order to minimize the risk of misplacement of the donor cell. The newly developed cloning technique, named as multiple Somatic Cell Nuclear Transfer (mSCNT), targeted to increase the potential for the donor fin cell to be placed in a favorable position inside the recipient sterlet egg and potentially to be reprogrammed. Comparing the single-SCNT and the mSCNT techniques, the later using interspecific mSCNT produced a higher percentage of early developing NTs (x 5.5), even though the surviving embryos did not develop as far as in our former study (Fatira et al., 2018). Besides, mSCNT was more effective than single-SCNT since the production of NTs was double in half the time of the SCNT technique. Time of operation is very important during animal cloning because both donor cells' and recipient eggs' quality is decreasing with the time and this results in cloning efficiency. The highlight of the mSCNT technique was the production of a gastrula stage multi nuclear transplant (mNT) of only donor's origin and two gastrula stage mNTs bearing both the recipient and donor genome. Even though the embryos arrested in gastrula it is not undermine the fact that it is the first report of donor's genome integration into embryos after sturgeon SCNT. This unique result after sturgeon SCNT is providing evidence for the potential regeneration of populations of large animals with high commercial and ecological value by iSCNT.

After sturgeon cloning, intraspecific or interspecific, single or multi SCNT, all developing NTs displayed development similar to the pattern of the sterlet control embryos (Fatira et al., 2018; Fatira et al., 2019). All sturgeon transplants that showed initial cleavage furrows continued development through the mid-blastula stage and many of them developed up to the gastrula stage. However, the proportion of blastula mNTs that gastrulated was generally lower than seen with NTs (Fatira et al., 2018). Therefore, the study suggested that the critical stage in sturgeon cloning could be to reach the early gastrula because all developing NTs that showed initial cleavage fulfilled the blastula. In previous studies conducted in teleost SCNT have been shown that a high percentage of the NTs face difficulties to overcome the mid-blastula (Siripattarapravat et al., 2009b; Luo et al., 2011; Le Bail et al., 2010), and it was attributed to be the stage at which the mitotic checkpoints are established (Ikegami et al., 1997), and at which the embryonic genome is activated (Kane and Kimmel, 1993). Failure to develop further than the mid-blastula stage is inferred to the incomplete epigenetic resetting of the donor nucleus in the egg environment that results in inaccurate zygotic gene expression (Luo et al., 2011). Before this stage, embryos develop with maternally supplied factors (Blelloch et al., 2006; Niemann et al., 2008). In Russian sturgeon the embryonic genome is activated in the late blastula (Dettlaff et al., 1993) and it is reasonable to assume that this applies to all sturgeons. In amphibian SCNT, the critical stage seems to be the late blastula (Gurdon, 1962; Gurdon et al., 1975), as demonstrated by Dettlaff et al. (1993) who noted the similarity in egg structure and cleavage pattern of acipenserids and amphibians. This similarity is also expressed in the process of gastrulation, the changes in the morphology of the embryo, morphogenetic movements, and the fate map of acipenserids being similar to those of anurans (Ballard, 1981). In future studies, before sturgeon SCNT would be necessary to standardize the cell-type to be used as donor-nucleus and after sturgeon SCNT would be important to check the ploidy of blastula NTs in addition to parentage assignments for detecting the contribution of donor and recipient nuclei. Furthermore, detailed histological observation during the first cell stage developing NTs could be performed to investigate how donor cell(s) is/are contributed in the recipient egg as well as transcriptome profiles during the embryonic development. Probably, in part, the observation of plastic sections and donor nuclear staining with DAPI would be efficient for these observations.

In both studies, non-enucleated and non-activated sterlet eggs were utilized (Fatira et al., 2018; 2019), which, in teleost SCNT, has been shown to have a positive result in goldfish (Le Bail et al., 2010). One of the five-gastrula stage Russian sturgeon mNTs possessed only Russian sturgeon donor alleles and that was the first report of a Russian sturgeon, a critically endangered species, generated from iSCNT. This exclusively donor-DNA-derived embryo was evidence of the potential of assisted reproduction technology to conserve the threatened population by iSCNT (Wilmut et al., 1997; Kishigami et al., 2008; Loi et al., 2013). Recently, has been proposed a mechanism after godlfish SCNT that the egg DNA remains under the first cleavage grove and is scattered without replication in the blastomeres upon successive cleavage, or that it is extruded as a whole with the second polar body, because of an alteration of the meiotic furrow (Rouillon et al., 2019). The limitation of the present study was that the Russian sturgeon mNT ceased development in the embryonic phase. As already discussed in teleost (Chênais et al., 2019), fin cells bear a specific differentiated profile based on epigenetic marks which may not be accurately reprogrammed during SCNT, such as numerous genes that are differentially expressed between embryos obtained after fertilization and after the nuclear transfer (Luo et al., 2009).

Another interesting result is that after the interspecific mSCNT between the Russian sturgeon and sterlet, 40% of the gastrula stage Russian sturgeon mNTs contained both recipient and donor genome, the first donor DNA integration reported in the embryo with sturgeon SCNT. This can be explained by the use of non-enucleated eggs. In medaka (Niwa et al., 1999, 2000; Wakamatsu and Ozato, 2002; Bubenshchikova et al., 2005) and in zebrafish cloning (Hattori et al., 2011), after single blastula nuclei transplant into non-enucleated unfertilized eggs, the NTs that grew to the adult stage expressed genetic markers of both donor and recipient.

Last but not least, it is interesting to emphasize that the sterlet eggs showed high resistance to stressful conditions. Despite the "rough" manipulation due to microinjection, and the stress of multiple fin cell injection, most mNTs that showed early development reached to the gastrula even when an injury from the microinjection position was visible. This affirms the suggestion of Ginsburg and Dettlaff (1991) of the value of sturgeon species for experimental embryological studies.

The present Ph.D. study provides evidence that interspecific cloning can be used for the reproduction of critically endangered large animals. Starting from establishing the basic steps of SCNT according to sturgeon's biology and subsequently improving the cloning technique according to species' limitations, the present study represents a comprehensive work. Future applications of the cloning technique could be utilized in selective breeding programs for sturgeon species even sturgeon individuals with favorable characteristics like big belly cavity and therefore bigger production of caviar. Some researchers have suggested that as long as somatic cells are cryopreserved, live animals (including fish) can be regenerated using nuclear transplantation technology (Mastromonaco et al., 2014). However, only nucleus-cytoplasm hybrids carrying mitochondrial DNA derived from the recipient species can be produced, and these animals are not suitable for return into wild environments.

In the future, if sturgeon SCNT will be widely used in fisheries industries will be suitable for caviar consumption and not for releasing sturgeons in the wild. In combination with an international protection policy of the natural environments of sturgeons, the species could be recovered.

Conclusions

The present Ph.D. thesis includes two scientific publications describing wherever the animal cloning technique can be applied to sturgeons, the limitations of the method as well as a significant improvement were investigated.

The main conclusions from these studies are:

1. The SCNT technique can be applied in sturgeon species. This is of high significance because large critically endangered rare animals can be generated via the powerful iSCNT technique.
2. The application of SCNT in sturgeons showed that there are not significant differences in transplants' development after intraspecific or interspecific cloning. This can be attributed to the high hybridization rate between the sturgeon species.
3. Sturgeon eggs are highly resistant in "rough" manipulations and can be used for extensive embryological studies.
4. After interspecific mSCNT have been obtained one specimen of donor origin only, while two specimens contained both recipient and donor genome. These findings are the first report of donor genome integration into a sturgeon embryo after interspecific cloning.

References

Ballard, W.W., 1981. Morphogenetic movements and fate maps of vertebrates. Am. Zool. 21, 391–399.

Blelloch, R. Wang, Z., Meissner, A., Pollard, S., Smith, A., Jaenischa, R., 2006. Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells 24, 2007–2013.

Birstein, V.J., Vasil'ev, V.P., 1987. Tetraploid-octoploid relationships and karyological evolution in the order Acipenseriformes (Pisces): karyotypes, nucleoli, and nucleolus-organizer regions in four acipenserid species. Genetica 73, 3–12.

Bubenshchikova, E., Ju, B., Pristyazhnyuk, I., Niwa, K., Kaftanovskaya, E., Kinoshita, M., Ozato, K., Wakamatsu, Y., 2005. Generation of fertile and diploid fish, medaka (*Oryzias latipes*), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. Cloning Stem Cells 7, 255–264.

Bubenshchikova, E., Kaftanovskaya, E., Motosugi, N., Fujimoto, T., Arai, K., Kinoshita, M., Hashimoto, H., Ozato, K., Wakamatsu, Y., 2007. Diploidized eggs reprogram adult somatic cell nuclei to pluripotency in nuclear transfer in medaka fish (*Oryzias latipes*). Dev. Growth Differ. 49, 699–709.

Chênais, N., Depincé, A., Le Bail, P.Y., Labbé, C., 2014. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of finfish genetic resources. Aquac. Int. 22, 63–76.

Chênais, N., Lorca, T., Morin, N., Guillet, B., Rime, H., Le Bail, P.Y., Labbé, C., 2019. Nuclear import of *Xenopus* egg extract components into cultured cells for reprogramming purposes: a case study on goldfish fin cells. Sci. Rep. 9, 2861.

Dettlaff, T.A., Ginsburg, A.S., Schmalhausen, O.I., 1993. Cleavage and Blastulation. In: Dettlaff, T.A., Ginsburg, A.S., Schmalhausen, O.I., (Eds.), Sturgeon Fishes. Developmental Biology and Aquaculture, Springer, Verlag, Berlin, pp. 92–105.

Fatira, E., Havelka, M., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T., 2018. Application of interspecific Somatic Cell Nuclear Transfer (iSCNT) in sturgeons and an unexpectedly produced gynogenetic sterlet with homozygous quadruple haploid. Sci. Rep. 8, 5997.

Fatira, E., Havelka, M., Labbé, C., Depincé, A., Pšenička, M., Saito, T., 2019. A newly developed cloning technique in sturgeons; an important step towards recovering endangered species. Sci. Rep. 9, 10453.

Ginsburg, A.S., Dettlaff, T.A., 1991. The Russian Sturgeon *Acipenser Güldenstadtii*. Part I. Gametes and early development up to time of hatching. In: Dettlaff, T.A., Vassetzky, S.G., (Eds.) Animal Species for Developmental Studies, Springer, Boston, MA, pp. 217–263.

Gurdon, J.B., 1962. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J. Embryol. Exp. Morphol. 10, 622–640.

Gurdon, J.B., Laskey, R.A., Reeves, O.R., 1975. The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. J. Embryol. Exp. Morphol. 34, 93–112.

Hattori, M., Hashimoto, H., Bubenshchikova, E., Wakamatsu, Y., 2011. Nuclear transfer of embryonic cell nuclei to non-enucleated eggs in zebrafish, *Danio rerio*. Int. J. Biol. Sci. 7, 460–468.

Havelka, M., Hulak, M., Bailie, D.A., Prodöhl, P.A., Flajšhans, M., 2013. Extensive genome duplication in sturgeons: New Evidence from Microsatellite Data. J. Appl. Ichthyol. 29, 704–708.

Havelka, M., Fujimoto, T., Hagihara, S., Adachi, S., Arai, K., 2017. Nuclear DNA markers for identification of Beluga and Sterlet sturgeons and their interspecific Bester hybrid. Sci Rep. 7, 1694.

Hochleithner, M., Gessner, J., 2012. The Sturgeons and Paddlefishes (Acipenseriformes) of the World – Biology and Aquaculture. In: Hochleithner, M., Gessner, J., (Eds.), The Sturgeons and Paddlefishes (Acipenseriformes) of the World, AquaTech Publications, Kitzbühel, Austria, pp. 178–190.

Ikegami, R., Rivera-Bennetts, A.K., Brooker, D.L., Yager, T.D., 1997. Effect of inhibitors of DNA replication on early zebrafish embryos: evidence for coordinate activation of multiple intrinsic cell-cycle checkpoints at the mid-blastula transition. Zygote 5, 153–175.

Kane, D.A., Kimmel, C.B., 1993. The zebrafish midblastula transition. Development 119, 447–456.

King, T.L., Lubinski, B.A., Spidle, A.P., 2001. Microsatellite DNA variation in Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus*: and cross-species amplification in the Acipenseridae. Conserv. Genet. 2, 103–119.

Kishigami, S., Wakayama, S., Hosoi, Y., Iritani, A., Wakayama, T., 2008. Somatic cell nuclear transfer: Infinite reproduction of a unique diploid genome. Exp. Cell Res. 314, 1945–1950.

Le Bail, P.Y. Depincé, A., Chênais, N., Mahe, S., Maisse, G., Labbé, C., 2010. Optimization of somatic cell injection in the perspective of nuclear transfer in goldfish. BMC Dev. Biol. 10, 64.

Lee, K.Y., Huang, H., Ju, B., Yang, Z., Lin, S., 2002. Cloned zebrafish by nuclear transfer from long-term-cultured cells. Nat. Biotechnol. 20, 795–799.

Liu, T.M., Yu, X.M., Ye, Y.Z., Zhou, J.F., Wang, Z.W., Tong, J.G., Wu, C.J., 2002. Factors affecting the efficiency of somatic cell nuclear transplantation in the fish embryo. J. Exp. Zool. 293, 719–725.

Loi, P., Iuso, D., Czernik, M., Zacchini, F., Ptak, G., 2013. Towards storage of cells and gametes in dry form. Trends Biotechnol. 31, 688–695.

Luo, D., Hu, W., Chen, S., Xiao, Y., Sun, Y., Zhu, Z., 2009. Identification of differentially expressed genes between cloned and zygote-developing zebrafish (*Danio rerio*) embryos at the dome stage using suppression subtractive hybridization. Biol. Reprod. 80, 674–684.

Luo, D.J., Hu, W., Chen, S.P., Zhu, Z.Y., 2011. Critical developmental stages for the efficiency of somatic cell nuclear transfer in zebrafish. Int. J. Biol. Sci. 7, 476–486.

Mastromonako, G.F., González-Grajales, L.A., Filice, M., Comizzoli, P., 2014. Somatic cells, stem cells and induced pluripotent stem cells: how do they now contribute to conservation? Repr. Scie. An. Conserv. 753, 385–427.

May, B., Krueger, C.C., Kincaid, H.L., 1997. Genetic variation at microsatellite loci in sturgeon: primer sequence homology in *Acipenser* and *Scaphirhynchus*. Can. J. Fish Aquat. Sci 54, 1542–1547.

McQuown, E., Gall, G.A.E., May, B., 2002. Characterization and inheritance of six microsatellite loci in lake sturgeon. Trans. Am. Fish Soc. 131, 299–307.

Niemann, H., Tian, X.C., King, W.A., Lee, R.S.F., 2008. Epigenetic reprogramming in embryonic and foetal development upon somatic cell nuclear transfer cloning. Reproduction 135, 151–163.

Niwa, K., Ladygina, T., Kinoshita, M., Ozato, K., Wakamatsu, Y., 1999. Transplantation of blastula nuclei to non-enucleated eggs in the medaka, *Oryzias latipes*. Dev. Growth. Dif. 41, 163–172.

Niwa, K., Kani, S., Kinoshita, M., Ozato, K., Wakamatsu, Y., 2000. Expression of GFP in nuclear transplants generated by transplantation of embryonic cell nuclei from GFP-transgenic fish into nonenucleated eggs of medaka, *Oryzias latipes*. Cloning 2, 23–34.

Rouillon, C., Depincé, A., Chênais, N., Le Bail, P.Y., Labbé, C., 2019. Somatic cell nuclear transfer in non-enucleated goldfish oocytes: understanding DNA fate during meiosis resumption and first cellular division. Sci. Rep. 9, 12462.

Siripattarapravat, K., Busta, A., Steibel, J.P., Cibelli, J., 2009a. Characterization and *in vitro* control of MPF activity in zebrafish eggs. Zebrafish 6, 97–105.

Siripattarapravat, K., Pinmee, B., Venta, P.J., Chang, C.C., Cibelli, J.B., 2009b. Somatic cell nuclear transfer in zebrafish. Nat. Methods 6, 733–735.

Sohrabnezhad, M., Kalbassi, M.R., Nazari, R.M., Bahmani, M., 2006. Short-term storage of Persian sturgeon (*Acipenser persicus*) ova in artificial media and coelomic fluid. J. Appl. Ichthyol. 22, 395–399.

Tung, T.C., Wu, S.C., Tung, Y.Y.F., Yen, S.S., Tu, M., Lu, T.Y., 1963. Nuclear transplantation in fishes. Sci. Sin. 14, 1244–1245.

Fontana, F., Lanfredi, M., Rossi, R., Bronzi, P., Arlati, G., 1996. Karyotypic characterization of *Acipenser gueldenstaedti* with C-, $AgNO_3$ and fluorescence banding techniques. Ital. J. Zool. 63, 113–118.

Wakamatsu, Y., Ozato, K., 2002. Cloning of Fish. In: Cibelli, J., Lanza, R., Campbell, K., West, M., (Eds.), Principles of Cloning. Second Edition, Academic Press, Elsevier, London, UK, pp. 287–299.

Wakamatsu, Y., 2008. Novel method for the nuclear transfer of adult somatic cells in medaka fish (*Oryzias latipes*): use of diploidized eggs as recipients. Dev. Growth Differ. 50, 427–436.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H.S., 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810–813.

English summary

Nuclear transplantation in sturgeon eggs

Effrosyni Fatira

The development of reproductive biotechnology is opening a new window for the conservation of threatened wildlife, as a backup when all other protection policies have failed. In this sense, nuclear transfer, also called cloning, is expected to be a useful tool to preserve species that are nearly extinct or to reconstruct extinct species. Interspecific somatic cell nuclear transfer (iSCNT) application to endangered sturgeon species has a great advantage, as the reconstruction of the critically threatened species can be achieved after a single fin-cell is transplanted in the egg-cytoplasmic environment of species whose eggs are easily available in farms. In the present Ph.D. study, the sterlet, considered to be a model species for sturgeon family, has been used as the egg recipient while the Russian sturgeon and the beluga, considered to be mostly favorable for caviar consumption, as well as the albino sterlet, have been used as donor fin cells.

Overall, the SCNT methodology was a very delicate multi-step procedure that required optimization of many experimental conditions with precise techniques and skillful manipulations. In this study, the crucial steps of sturgeon cloning have been tested by adjustment of the experimental conditions with intraspecific and interspecific SCNT. The study demonstrated that the iSCNT can be applied to real endangered species. In addition, after the improvement of the iSCNT technique by utilizing the mSCNT, the present study could obtain for the first time a specimen (0.8%) from the donor's origin only, while two specimens (1.6%) showed both the recipient and donor genome. These results were of high significance because the donor DNA was able to integrate into a sturgeon embryo after interspecific cloning.

In all, the present Ph.D. study provides evidence that cloning with the multiple donor somatic cells can be feasible in the future of aquaculture used for meat and caviar consumption. Despite the fact that sturgeon cloning faces limitations, to date it is a promising technique for their preservation together with international protection policies that will protect the habitat of wild sturgeon population.

Czech summary

Jaderná transplantace u jiker jesetera

Effrosyni Fatira

Vývoj reprodukční biotechnologie otevírá nové možnosti pro ochranu ohrožených živočichů, je tak zálohou v případě selhání všech ostatních způsobů ochrany. Jednou z takových biotechnologií je jaderný transfer, nazývaný také klonování, který se může stát užitečným nástrojem k zachování druhů, které jsou téměř zaniklé nebo k obnovení druhů již zaniklých. Aplikace interspecifických jaderných transferů somatických buněk (SCNT) na ohrožené druhy jeseterů má velkou výhodu. Obnovení kriticky ohrožených druhů lze totiž dosáhnout po transplantaci jediné buňky z ploutve do cytoplazmy vajíček druhů, které jsou snadno dostupné na farmách. V prezentované studii byl jeseter malý, považovaný za modelový druh jesetera, použit jako recipient vajíček. Zatímco jeseter ruský a vyza velká, kteří jsou považováni za kriticky ohrožené druhy, a albinotická forma jesetera malého byli použiti jako donoři buněk získaných z ploutví.

Celkově je metodika SCNT velmi delikátním vícekrokovým postupem, který vyžaduje optimalizaci mnoha experimentálních podmínek, precizní techniky a manipulace. V této studii byly klíčové kroky klonování jeseterů optimalizovány pro intraspecifický a interspecifický SCNT. Studie prokázala, že SCNT lze skutečně aplikovat i na ohrožené druhy. Kromě toho jsme po vylepšení techniky SCNT pomocí injikace více somatických buněk dokázali poprvé získat jedince (0,8 %) pouze s původem donora, zatímco dva vzorky (1,6 %) vykazovaly jak genom recipienta, tak donora. Tyto výsledky jsou velmi významné, neboť jsme tím dokázali, že donorová DNA byla schopna integrace do embrya jesetera po interspecifickém klonování.

Tato dizertační práce prokázala, že klonování prostřednictvím více somatických buněk donora může být v budoucnosti proveditelné i pro potřeby akvakultury za účelem produkce masa a kaviáru. Navzdory jistým omezením spojených s jadernýn transferem jeseterů se stále jedná o slibnou techniku pro jejich zachování, která doplňuje mezinárodní regulace a úmluvy pro ochranu ekosystémů, ve kterých jeseteři žijí.

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- Grant from the Czech Science Foundation (grant number 17-19714Y).
- One-month scholarship from Developmental Project for foreign internship in Ehime University, Ainan town, Japan under the supervision of Dr. Taiju Saito.
- One - year individual grant project from the Grant Agency of the University of South Bohemia in České Budějovice, GAJU 126/2015/Z Fatira.
- One - year individual grant project from the Grant Agency of the University of South Bohemia in České Budějovice, GAJU 079/2016/Z Fatira.

- Two-months scholarship from Developmental Project for foreign internship in Ehime University, Ainan town, Japan under the supervision of Dr. Taiju Saito.
- One - year individual grant project from the Grant Agency of the University of South Bohemia in České Budějovice, GAJU 068/2017/Z Fatira.
- Registration – Grant for attending the 'Fourth World Congress of Reproductive Biology, (WCRB2017) in Okinawa, Japan.

List of publications

Peer-reviewed journals with IF

Fatira, E., Havelka, M., Labbé, C., Depincé, A., Pšenička, M., Saito, T., 2019. A newly developed cloning technique in sturgeons; an important step towards recovering endangered species. Scientific Reports 9, 10453. (IF 2018 = 4.011)

Fatira, E., Havelka, M., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T., 2018. Application of interspecific Somatic Cell Nuclear Transfer (iSCNT) in sturgeons and an unexpectedly produced gynogenetic sterlet with homozygous quadruple haploid. Scientific Reports 8, 5997. (IF 2018 = 4.011)

Abstracts and conference proceedings

Fatira, E., Labbé, C., Depincé, A., Iegorova, V., Pocherniaieva, K., Güralp, H., Havelka, M., Pšenička, M., Saito, T., 2017. Single and multiple somatic cells nuclear transfer in critically endangered species, Sturgeon. "The 6th International Workshop on the Biology of Fish Gametes (IWBFG)", 4–7 September 2017, **České** Budějovice, Czech Republic (**Oral presentation**).

Fatira, E., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T., 2017. Interspecies single somatic cell nuclear transplantation technique in Sturgeon. International Symposium in "Fisheries Science for Future Generations", The Japanese Society of Fisheries Science, (JSFS), 22–24 September 2017, Tokyo, Japan (**Oral presentation**).

Fatira, E., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T., 2017. Somatic cell nuclear transfer in a real endangered species, Sturgeon. "The 4th World Congress of Reproductive Biology (WCRB)", 27-29 September 2017, Okinawa, Japan (**Oral & poster presentation**).

Fatira, E., Pšenička, M., Saito, T., 2017. Single or multi-intra-cytoplasm sperm injection techniques among Sturgeon species. "The 6th International Workshop on the Biology of Fish Gametes (IWBFG)", 4–7 September 2017, **České** Bud**ě**jovice, Czech Republic (**Poster presentation**).

Fatira, E., Pšenička, M., Arai, K., Saito, T., 2015. Nuclear transfer in sterlet eggs: a first attempt. "The 5th International Workshop on the Biology of Fish Gametes (IWBFG)", 7–11 September 2015, Ancona, Italy (**Poster presentation**).

Training and supervision plan during study

Name	Effrosyni Fatira	
Research department	2014–2019 – Laboratory of Germ cells of FFPW	
Supervisor	Taiju Saito, Ph.D.	
Period	29th September 2014 until 22nd June 2020	

Ph.D. courses	**Year**
Basic of scientific communication	2015
Fish reproduction	2015
Applied hydrobiology	2015
Pond aquaculture	2016
Ichthyology and fish taxonomy	2016
English language (FCE)	2016
Czech language	2017

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2014 2015 2016 2017

Scientific seminars	**Year**
Fatira, E., Pšenička, M., Arai, K., Saito, T. Nuclear transfer in sterlet eggs: a first attempt. "The 5th International Workshop on the Biology of Fish Gametes (IWBFG)", 7–11 September, Ancona, Italy	2015
Fatira, E., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T. Somatic cell nuclear transfer in a real endangered species, Sturgeon. "The 4th World Congress of Reproductive Biology (WCRB)", 27–29 September, Okinawa, Japan.	2017
Fatira, E., Labbé, C., Depincé, A., Iegorova, V., Pšenička, M., Saito, T. Interspecies single somatic cell nuclear transplantation technique in Sturgeon. International Symposium in "Fisheries Science for Future Generations", The Japanese Society of Fisheries Science, (JSFS), 22–24 September, Tokyo, Japan.	2017
Fatira, E., Labbé, C., Depincé, A., Iegorova, V., Pocherniaieva, K., Güralp, H., Havelka, M., Pšenička, M., Saito, T. Single and multiple somatic cells nuclear transfer in critically endangered species, Sturgeon. "The 6th International Workshop on the Biology of Fish Gametes (IWBFG)", 4–7 September, České Budějovice, Czech Republic.	2017
Fatira, E., Pšenička, M., Saito, T. Single or multi-intra-cytoplasm sperm injection techniques among Sturgeon species. "The 6th International Workshop on the Biology of Fish Gametes (IWBFG)", 4–7 September, České Budějovice, Czech Republic.	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	Year
Dr. Catherine Labbé, Institut National de la Recherche Agronomique (INRA), Rennes, France (1 month, learning the single somatic cell nuclear transfer technique in a model species, goldfish, *Carassius auratus*).	2015
Dr. Catherine Labbé, Institut National de la Recherche Agronomique (INRA), Rennes, France (10 days, analysis of goldfish spermatozoa flagella movement).	2015
Dr. Katsutoshi Arai, Hokkaido University, Hakodate, Japan (1 month, performing multi-cell injection into oocytes using as a fish model the zebrafish, *Danio rerio*).	2015
Dr. Taiju Saito, South Ehime University, Ainan, Japan (1 month, investigating the effects in the embryonic pattern of medaka after multi-cell transfer).	2015
Dr. Taiju Saito, South Ehime University, Ainan, Japan (3 months, performing multiple fin cells injection into fish embryos).	2016
Dr. Goro Yoshizaki, Tokyo University of Marine Science and Technology (TUMSAT) (1 month, performing spermatogonia transplantation techniques in zebrafish, medaka and bitterling).	2017

Pedagogical activities	Year
Lecturing and training of student of bachelor study, transplantation technique in zebrafish, incubation and evaluation of the resulting embryos in range of 90 teaching hours.	2015
Leader of project during summer school entitled: “A novel nuclear transplantation technique on zebrafish; methods and evaluation”.	2017
Announcing the project at summer school entitled: “Nuclear transplantation in fish; methods and evaluation”.	2018

Curriculum vitae

PERSONAL INFORMATION

Name: Effrosyni
Surname: Fatira
Title: M.Sc.
Born: 10th April, 1988, Ioannina, Greece
Nationality: Greek
Languages: Greek, English, Italian, French, Czech, Spanish
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EDUCATION

2014–2019 Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic

2011–2013 M.Sc. in Biology-Management of Terrestrial and Marine Biological Resources at the University of Crete, Heraklion, Greece
Dissertation title: Comparative induction of spawning success in meagre (*Argyrosomus regius*) using GnRHa injections or implants and monitoring of egg quality (Supervisor: Dr. Constantinos C. Mylonas)

2006–2011 B.Sc. in Biology, Department of Biology, University of Crete, Heraklion, Greece
Dissertation title: Effect of body size and stress intensity in cortisol response in European sea bass, *Dicentrarchus labrax* (Supervisor: Dr. Michalis Pavlidis)

TRAINING

05/2015 The Epiconcept Training School in Epigenetics in Reproductive Biology, Murcia, Spain. Attending both theoretical and practical sessions in facilities from Veterinary Schools as well as from the Central Services for Research Support located on Campus

07–08/2015 Period of training in Experimental Fish Culture Facility (modelling), Faculty of Fisheries and Protection of the Waters, Vodnany, Czech Republic

06–07/2015 Period of training in Genetic Fisheries Center, Faculty of Fisheries and Protection of the Waters, Vodnany, Czech Republic

06/2016 6th AQUAGAMETE Training School, Fish Physiology and Genomics, Department of INRA, Rennes, France. Learning how to use Genomic tools in order to evaluate fish gamete's quality

RESEARCH STAY AND COLLABORATIONS

02–03/2015 Dr. Catherine Labbé, Institut national de la recherche agronomique (INRA), Rennes, France

04–05/2015 Dr. Catherine Labbé, Institut national de la recherche agronomique (INRA), Rennes, France

10–11/2015 Dr. Katsutoshi Arai, Hokkaido University, Hakodate, Japan

12/2015 Dr. Taiju Saito, Ehime University, Nishiura Station, South Ehime Fisheries Research Center

10–12/2016 Dr. Taiju Saito, Ehime University, Nishiura Station, South Ehime Fisheries Research Center

10/2017 Dr. Goro Yoshizaki, Tokyo University of Marine Science and Technology (TUMSAT), Tokyo, Japan