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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Sturgeons

Sturgeons are one of the oldest fish families known as living fossils. They are source of one of the most expensive food products, viz. black caviar. This group includes the genera *Acipenser, Huso, Scaphirhynchus*, and *Pseudoscaphirhynchus* from the family Acipenseridae (sturgeons), as well as two species from the family Polyodontidae (paddlefishes) *Psephurus gladius* and *Polyodon spathula*. Altogether 27 extant sturgeon species inhabit the whole northern hemisphere along the coast of the Pacific and Atlantic oceans, seas, inland lakes and rivers. These species represent one of the most highly imperiled groups of taxa, with a worldwide reduction in abundance and distributions common among most species (Birstein et al., 1997; Rosenthal et al., 2006; IUCN, 2011). Recent reports suggested that 85% of the world's sturgeon species are currently at risk of extinction, making them the most threatened group of fish on the IUCN Red List of Threatened Species (IUCN, 2011). This depletion of wild population is mostly caused by overexploitation (overfishing) and habitat degradation. The industrial production of sturgeon products began two centuries ago. Till the beginning of the 20th century, the United States was the main exporter of black caviar, mainly roe of *Acipenser oxyrinchus* (Birstein et al., 1997; Secor, 2002). Shortly after the beginning of the 20th century, Russia took the lead and became a major caviar trading nation till end of the century when rapid reduction of wild populations and establishment of conservation programs decreased the amount of legal catches (Taylor, 1997; Secor et al., 2000). Lately, the Caspian Sea nations of Iran, Kazakhstan, Russia, and to a lesser extent Azerbaijan and Turkmenistan, dominated the international trade in capture fisheries products despite restriction of CITES regulations (Convention on International Trade in Endangered Species), which imposed limited export and catch quotas since 1997 when all commercially utilized sturgeon species were listed in Annex II as species that may became threatened with extinction (Bronzi et al., 2011; Hoover, 1998; Raymakers & Hoover, 2002). Despite strict regulations, illegal overexploitation still represents a huge threat to wild populations. Consequently, tight restrictions on the exploration of wild stock increased demands for aquaculture-produced black caviar (see Fig. 1.1.1.; Bronzi et al., 2011; Fontana et. al., 2001; Billard and Lecointre, 2001; Pikitch et al., 2005). Hence the future of black caviar production is heading towards massive development of sturgeon aquaculture resulting in reducing the fishing pressure on natural stocks (Bronzi et al., 2011).

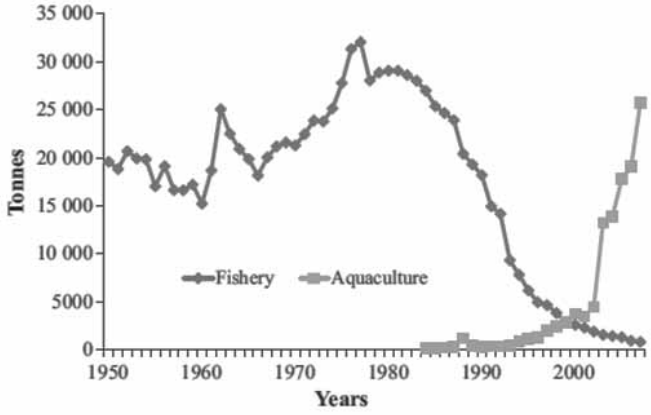

Fig. 1.1.1. *Comparison of the official statistics for global sturgeon catching and aquaculture of all sturgeon species, according to Bronzi et al. (2011).*

In the past decades, aquaculture production of sturgeon has been increasing. The low demands and prices for sturgeon meat compared with those for caviar have led to the formation of a unilateral market of sturgeon products focusing on caviar production. As a result, commercial aquaculture focused on methods of unisexual broodstock production and offspring separation by sex. Unfortunately, separation of sturgeon offspring according to sex is problematic because of the absence of phenotypically distinctive features. Moreover, till date, there is no final agreement on sturgeon sex determination because sex chromosomes, as well as sex-specific genetic markers have not been found (Keyvanshokooh & Gharaei, 2010; Wuertz et al., 2006). A commonly used practice is separation of females by performing a biopsy of the gonads, performing an endoscopy (Hurvitz et al., 2007), or using modern noninvasive ultrasound diagnostic (Chebanov and Galich, 2009), as well such plasma sex steroids analysis techniques (Van Eenennaam et al., 2004; Webb et al., 1999, 2001; Hurvitz et al., 2005). Such methods are hindered because of long periods of maturation of sturgeon progeny. Depending on species and farming conditions (e.g., water temperature, stock density, and feeding) maturation can last for few years (Chebanov and Galich, 2013). A new approach in sturgeon sexing has recently been developing and is based on studies of proteins involved in sex determination and differentiation at different stages of gonadal maturation (Keyvanshokooh et al., 2009). The costs of rearing males are very high compared with their costs in the meat market; thus, rearing males till sexing is usually nonprofitable. Therefore, methods allowing alterations in the sex ratio of sturgeon broodstock, such as chromosomal manipulations, are under meticulous investigation. Alternatively, hormonal stimulation of juvenile fish can be used for sex reversal of progeny, and thus obtaining a unisexual stock. Positive results of hormonal feminization in bester and Russian sturgeon was reported by Kovalev et al. (2012). Omoto (2002) reported successful feminization of bester hybrids in whom 97% of gonads differentiated into ovaries. This method was based on feeding fry a diet containing estradiol-17β. It is still unknown whether steroids affected the proliferation and/or development of oocytes, as well as whether these fish can mature to become functional females (Omoto et al., 2002). In addition, the strict legislative rules restrict the application of hormones in the food production industry (Stephany, 2010).

It is worth mentioning that sturgeons are not only important aquaculture resources because of caviar and boneless meat but are also a model animal for chromosomal manipulation and the study of ploidy-level evolution. Spontaneous polyploidization and hybridization between sturgeon species resulted in high complexity and plasticity of the ploidy system, making them a unique model for ploidy manipulation studies (Havelka et al., 2013). Chromosomal manipulation as well as hybridization studies play a key role in expanding our understanding of polyploidy nature and species evolution.

1.2. Chromosomal manipulations in aquaculture

Chromosomal manipulations involve artificial influence on gametes and/or zygotes directed at altering the chromosomal composition of the offspring. A few types of chromosomal manipulations exist, viz. ploidy manipulation, gynogenesis, and androgenesis. Combined with hybridization, these methods are an extremely powerful tool for breeding, manipulating the phenotype, as well as investigation the sex differentiation system and evolutional relationships among species (Pandian and Koteeswaran, 1998; Arai, 2001; Ihssen et al., 1990).

1.2.1. Ploidy manipulations

Successful artificial induction of polyploidy has been recorded in a variety of species (Pandian and Koteeswaran, 1998). This method is a powerful tool to manipulate the phenotype of a progeny and achieve traits such as sterility. In general, all triploids might be considered sterile (Piferrer et al., 2009). Therefore, triploidization techniques are often used for the production of sterile all-female stocks (e.g., rainbow trout, *Oncorhynchus mykiss*) to avoid problems associated with sexual maturation, such as lower growth rates and increased incidence of decease. In addition, triploidization is used for decreasing the possible genetic impact of farmed escapees on wild population (Piferrer et al., 2009). Although in rare species-specific cases, autotriploids can produce gametes capable of fertilization, hence making the prospect of using triploidization for biocontainment of farmed populations, at least as a sole precautionary measure, unreliable. Fertile polyploids, particularly tetraploids, can be used for further chromosomal manipulation such as the induction of androgenesis, gynogenesis, allo-/auto-triploidization (in rainbow trout, *Oncorhynchus mykiss*, by Chourrout et al., 1986; crucian carp, *Carassius carassius*, by Luo et al., 2011), or the production of fish with higher ploidy (in loach, *Misgurnus anguillicaudatus*, Arai et al., 1993). Allotriploidy induced by artificial interspecific hybridization followed by shock treatment to retain the second polar body can increase the viability with respect to diploid hybrids with a poor vitality (Scheerer and Thrgaad, 1983).

The methods of polyploidization in fish are based on the suppression of the second meiotic division (triploidization) or the first mitotic division (tetraploidization). Triploidization treatment is possible because fish eggs are released during metaphase of the second meiosis phase and further development is induced by the entry of a spermatozoon, leading to the extrusion of the second polar body (the second phase of meiosis). Physical or chemical shock treatment during this time can suppress the extrusion of the second polar body, making the zygote triploid, which bears one chromosome set of each the maternal and paternal pronuclei and the third chromosome set of the maternal second polar body (Pandian and Koteeswaran, 1998). In some cases, triploids can be obtained by fertilization of eggs by diploid sperms of tetraploid males, e.g., in mud loaches (*Misgurnus mizolepis*; Nam and Kim, 2004), sea basses (*Dicentrarchus labrax*; Francescon et al., 2004), and Pacific oysters (*Crassostrea gigas*; Guo and Allen, 1994). Furthermore, allotriploids can be produced by crossing two distantly related species, backcrossing the fertile F_1 interspecific hybrids with one of the paternal species (Arai, 1988; Benfey, 1989; Vrijenhoek et al., 1989; Pandian and Koteeswaran, 1998), or intercrossing closely related aquaculture species followed by triploidization, as in flatfishes, salmonids and sparids (Purdom, 1972; Chevassus, 1983; Gorshkov et al., 1998). Tetraploidization is based on the suppression of the first cleavage division that leads to duplication of the zygote chromosome number. Tetraploidy was induced in a substantially lower number of species, and at the end of the 20th century, viable tetraploid progeny were obtained in only six fish species (Pandian and Koteswaran, 1998). This method usually has a substantially lower efficiency and survival rate than triploidization.

Sturgeons have a very flexible ploidy system with possible hybridization between sturgeon species with different ploidy. This plasticity of the sturgeon ploidy system originates from possible hybridization of species with different ploidy levels and spontaneous polyploidization because of occasional failure of the extrusion of the second polar body of the fertilized egg (Fontana et al., 2001). In addition, in contrast to most teleost species, sturgeon triploids were reported to be fertile (Omoto et al., 2005; Drauch et al., 2011; Pšenička et al., 2011; Havelka et al., 2014). The complexity of the sturgeon ploidy system determination aggravates because of presence of a high number of dot-like microchromosomes, in some cases more

than half (Fontana et al., 2001; Birstein et al., 1997). Early investigation of sturgeon genome structure and size by karyotype or flow cytometry assorted extant sturgeon species into three groups: those possessing approximately 120 (3.2–4.6 pg), 250 (6.1–9.6 pg), and shortnose sturgeons (*Acipenser brevirostrum*) with 372 ± 6 (13.1 pg) chromosomes (Birstein et al., 1993; Blacklidge and Bidwell 1993; Fontana et al., 2008; Kim et al., 2005). These groups are called evolutionary tetraploid, octaploid, and dodecaploid, respectively (Flajshans & Vajcova, 2000). However, in 1983, Arefjev proposed the diploid origin of 120-chromosome species. Evidence for this hypothesis was provided by Fontana (1994). Havelka et al. (2013) classified sturgeon species into three groups: functional diploids, tetraploids, and hexaploid shortnose sturgeons. The functional hexaploidy origin of the shortnose sturgeon was confirmed by fluorescent in situ hybridization (FISH) by Fontana (2008) and by microsatellite analysis by Havelka et al (2013).

1.2.2. Androgenesis

Androgenesis is the type of unisexual reproduction where all nuclear genetic material transmitted to progeny from the father (Corley et al., 1996). This condition can arise naturally in fish and can be achieved artificially. For artificial androgenesis induction, eggs should be treated in order to inactivate maternal nuclear DNA. Ionizing or UV radiation is commonly used to induce DNA damage in eggs. Fertilization of these eggs leads to androgenetic homozygote development. Androgenetic homozygotes of sturgeons were first obtained by Grunina (1995). Although, such zygotes are initially haploid and normally die during embryonic or early larval stages, diploidy of androgenetic homozygotes can be restored by temperature or hydrostatic pressure shock treatment applied during the first cleavage division. Basically, the procedure of this shock is equal to that of tetraploidy induction. This process doubles the haploid chromosome set, but because individuals are entirely homozygous, the viability of induced androgenotes is usually low. Moreover, mitotic diploidy restoration usually has a low success rate and leads to malformations of larvae development. Restoration of diploidy in androgenotes by fusion of two sperm nuclei allows androgenetic progeny to have a heterozygosity level similar to that obtained in a regular crossing and such method is called dispermic androgenesis. This method includes insemination of genetically inactivated eggs with concentrated sperms (to cause polyspermy). Using this method, viable androgenetic progenies were obtained for the first time in the Siberian (*Acipenser baerii*), Russian (*Acipenser gueldenstaedtii*), stellate (*Acipenser stellatus*), and beluga (*Huso huso*) sturgeons (Grunina et al., 2006). Many authors suggest that this method may allow long term preservation of endangered species as cryopreserved sperm samples. In addition, a recent study by Grunina and Recoubratsky (2005) revealed the possibility of using heterologous sperms for the induction of so called androgenenetic hybrids. This method reveals new possibilities for restoring endangered species using females of close species. Another method of androgenote production is fertilizing irradiated eggs with diploid sperms produced either from tetraploid fish or by chemical fusion prior to fertilization (Devlin and Nagahama, 2002). This method of obtaining gynogenotes is particularly interesting in sturgeons because of their ploidy-level plasticity.

1.2.3. Gynogenesis

Gynogenesis is a chromosomal manipulation technique that leads to the inheritance of solely maternal genetic material, and this has been previously accomplished in many fish species (Ihssen et al., 1990; Pandian and Koteeswaran, 1998). Gynogenetic offspring are produced by the same mechanism as that in spontaneous parthenogenesis, but the egg

should be stimulated by the presence of sperms in order to develop. Natural gynogenesis has been described in several all-female fish forms (Ihssen et al., 1990; Pandian and Koteeswaran, 1998), which parasite on natural reproduction of a related species. Their sperm triggers the egg development, and thus genetic information of the male pronucleus is not incorporated in further development. Therefore, diploid gynogenotes possess two copies of the maternally inherited chromosome set (Pandian and Koteeswaran, 1998; Beaumont and Hoare, 2003; Flajshans, 2006). Artificial gynogenesis for was induced first time in the fish species brown trout by Opperman (1913). Since then, gynogenetic progeny of many different species were obtained. Particular interest was focused on cyprinids, Salmonids, Ictalurids, and Acepenserids families (Thorgaard, 1983; Benfley, 1989; Ihssen et al., 1990). The main purpose of gynogenesis induction in these commercially important fish species was to shift the sex ratio in progeny (Stanley et al., 1975), obtain inbred lines (Nagy et al., 1984), or was to investigate the sex determination system. The success rate of gynogenesis induction greatly varies with species. Usually, the sperm motility system sensitivity is the main limiting factor during gynogenesis induction. Furthermore, application of the commonly used UV method of sperm DNA inactivation leads to a great variability in experimental setups and proposed optimal doses, mainly because of low sperm transparency and consequently the necessity of sperm dilution in species with a high sperm density. In addition, several parameters of sperms such as the amount of DNA, membrane lipid content, the presence of sunscreen-like substances, antioxidants, and the level of photoreactivation may possibly affect the UV inactivation of DNA in sperm, leading to the variability of UV-irradiation parameters (Table 1.2.1.). Also, it is worth mentioning that the existing problem of relevant literature reporting the use of different units with regard to the UV dose, whereas parameters like the depth of the sperm layer, dilution of sperm, and intensity of UV light are often omitted, hampers comparisons of new protocols and casts doubts on the unconventional parameters of proposed protocols.

Table 1.2.1. *Parameters of protocols of UV irradiation for gynogenesis induction in different species compare different genome sizes. Haploid DNA contents (C-values, picograms of DNA per nucleus) according to the Animal Genome Size Database (www.genomesize.com)*

Species	Genome size, C-value (pg DNA nucleus^{-1})	Dilution (% of sperm in medium)	UV dose (J/m^2)	Author (year)
Paralichthys olivaceus	0.71	2.0	3600	Feng (2008)
Hippoglossus hippoglossus	0.73	1.25	650	Tvedt (2006)
Dicentrarchus labrax	0.78	5	3200	Peruzzi (2000)
Dicentrarchus labrax	0.78	1	330	Francescon (2004)
Scophthalmus maximus	0.86	9.1	3000	Piferrer (2004)
Esox lucius	0.9	10	768	Luczynsky (2007)
Morone chrysops	0.9	---	1000	Gomelsky (2000)
Perca flavescens	0.92	10.0	324	Malison (1993)
Perca flavescens	0.92	10	1719	Rinchard (2002)
Perca flavescens	0.92	10	1248	Dabrowski (2000)
Gadus morhua	0.93	2.5	900	Otterå (2011)
Oplegnathus fasciatus	0.93	2	300	Kato (2001)

Species	Genome size, C-value (pg DNA nucleus^{-1})	Dilution (% of sperm in medium)	UV dose (J/m^2)	Author (year)
Ctenopharyngodon idellus	1	5.3	4800	Fopp-Bayat (2009)
Oreochromis mossambicus	1	---	1764	Pandian & Varadaraj (1990)
Lateolabrax japonicus	1	10	300	Song-Lin (2009)
Oreochromis niloticus	1.12	---	198	Mair (1990)
Oreochromis niloticus	1.12	---	360	Hussain (1993)
Centropristis striata	1.2	10	700	Heidi (2009)
Ooreochromis aureus	1.21	---	1228	Don and Avtalion (1988)
Megalobrama amblycephala	1.20	25.0	36000	KaiKun (2011)
Esox masquinongy	1.28	10	1440	Lin (1996)
Leiciscus idus	1.5	---	1920	Kucharczyk (2004a)
Abramis brama	1.37	---	1920	Kucharcyk (2004b)
Carassius auratus auratus	1.7	1.0	2000	Fujioka (1993)
Bester hybrid	1.8	10	2100	Omoto (2005)
Cyprinus carpio	1.8	20	42000	Sun (2006)
Megalobrama amblycephala	1.20	20	36000	Sun (2006)
Cyprinus carprio	1.8	---	300	Cherfas (1994) Ilyasova&Cherfas, 1978
Cyprinus carprio	1.8	25	132000	Komen (1988)
Cyprinus carprio	1.8	33.3	75600	Khan (2000)
Cyprinus caprpio	1.8	20.0	9000	Stanley (1976)
Cyprinus carpio	1.8	25	300	Zhang (2011)
Cyprinus carpio	1.8	---	800	Cherfas (1990)
Misgurnus anguillicaudatus	1.86	10	380	Nam (2000)
Acipenser ruthenus	1.87	5.0	135	Recoubratsky (2003)
Misgurnus anguillicaudatus	2.3	---	600	Suzuki (1985)
Acipenser stellatus	2.35	5.0	243	Recoubratsky (2003)
Oncorhynchus mykiss	2.6	20	33000	Colihueque (1992)
Oncorhynchus mykiss	2.6	50	70500	Thompson (1984)
Oncorhynchus mykiss	2.6	2.5	6225	Goryczko (1991)
Acipenser gueldenstaedtii	3.94	5	297	Recoubratsky (2003)
Acipenser schrenckii	4	25	2589	Zou (2011)
Acipenser baerii	4.15	10	288.75	Fopp-Bayat (2010)
Acipenser transmontanus	5.1	10	2160	Van Eenennam (1996a)
Acipenser brevirostrum	6.89	25	1200	Flynn (2006)

1.3. Gynogenesis in sturgeons

The first experiments on induced gynogenesis in sturgeons were conducted by Romashov et al. (1963). Since then, the induction of gynogenesis has been reported for many sturgeon species. The main goals of sturgeon gynogenesis studies are yet to investigate the sex

determination system and the production of female broodstock (Streisinger et al., 1981; Delvin and Nagahama, 2002; Komen and Thorgaard, 2007). Species with the male heterogametic sex determination system completely have female gynogenetic progeny. In contrast, sturgeons have mixed sex in gynogenetic progeny, indicating the presence of the female heterogametic sex determination system called the ZW/ZZ system. The hypothesis of female heterogamety in sturgeons is now commonly accepted (Recoubratsky et al., 2003; Omoto et al., 2005; Saber, 2014; Vasil'ev et al., 2010) and the corresponding percentage of females in gynogenetic progeny was shown in a number of sturgeon species: *Acipenser baerii*, 81% (Fopp-Bayat, 2010); *Acipenser transmontanus*, 82% (Van Eenennaam et al., 1999); Bester, 70%–80% (Omoto et al., 2005); and *Acipenser brevirostrum*, 65% (Flynn et al., 2006). In addition, it is expected that gynogenesis can help to primarily obtain female progeny because of the possible presence of WW superfemales in gynogenetic progeny. On the contrary, a few studies of sturgeons contradict this theory, such as Badrtdinov et al. (2008) who described complete male gynogenetic progeny of stellate sturgeon (*Acipenser stellatus*) and Grunina (2011) who found females in androgenetic progeny of Siberian sturgeon (*Acipenser baerii*). Moreover, there are only few reports on the actual growth and gonadal development of gynogenetic sturgeons; hence the application of gynogenesis induction in sturgeon aquaculture appears to be a distant prospect.

In general, the highest mortality of gynogenotes is observed during the first stages of embryogenesis (Van Eenennaam et al., 1996b; Omoto et al., 2005; Fopp-Bayat et al., 2007). Probably, the main reason for this is the low efficiency of egg activation caused by spermatozoa damage. The stochastic UV damage affects the spermatozoa volume including the motility system and/or acrosome. As a result, this damage decreases the ability of sperms to activate eggs. Thereby, many studies have focused on finding the compromise between full DNA inactivation and destruction of spermatozoa motility system and/or acrosome (Mims et al., 1995; Recoubratsky et al., 2003; Fopp-Bayat et al., 2007; Lebeda et al., 2014).

1.4. Inactivation of the paternal genome in sperms

Inactivation of genetic information in spermatozoa for activation of eggs is the most critical step in obtaining a gynogenetic progeny of sturgeons. This step is difficult to optimize because of the strong influence of DNA damaging agents on sperm motility and acrosome apparatus. A few ways to inactivate paternal DNA in sperms have been described, such as application of UV light, ionizing radiation, or chemical agents (Thorgaard, 1983; Ihssen et al., 1990; Felip et al., 2001).

1.4.1. UV-C irradiation

The most common way to inactivate DNA in spermatozoa is to irradiate them by short-wave UV light (called UV-C, 250 nm); however, studies on the influence of UV irradiation on the motility of sturgeon spermatozoa showed that their motility apparatus is highly sensitive to UV light (Recoubratsky et al., 2003; Dietrich et al., 2005). Furthermore, optimization of the UV treatment is complicated because of high optical density of sperms and significant difference in sperm density between males. Mims et al. (1995) proposed a way to optimize the UV dose for DNA inactivation in shovelnose sturgeons (*Scaphirhynchus platorynchus*). They suggested that 40% of the spermatozoa lethal dose is sufficient for paternal genome inactivation while saving spermatozoa moving activity. In 1996, Van Eenennaam suggested that the optimal UV dose depends on the unique developmental stage of the specific batch

of gametes involved in each cross. Moreover, different spermatozoa development stages of the released sperm as well as difference in the bloodstock diet can lead to variations in the lipid composition of the spermatozoa outer membrane making them more or less susceptible to UV induced ROS (Pustowka et al., 2000). Consequently, these variations increased the uncertainty of UV-irradiation protocol optimization, and thus warranting the adjustment of the protocols for each male.

1.4.2. X-ray and Gamma irradiation

These methods of paternal DNA inactivation were used at the beginning of gynogenesis studies. Ionizing irradiations damage DNA very effectively but also induce substantial damage to other systems of spermatozoon. The presence of residual chromosomal fragments in the karyotype of gynogenetic progeny produced by this method was reported in 1982 by Chourrout and Quillet, as well as intergeneric gene transfer in gynogenetic progeny of rainbow trout after the application of ionizing irradiation was described (Disney et al., 1987). In addition, this method is difficult to handle and expensive to set up. This method can be used for larger volumes of sperm samples during mass production of gynogenotes because of the high permeability of ionizing irradiations (Ihssen et al., 1990), or used for androgenesis induction in fish with large or nontransparent eggs (Arai et al., 1979).

1.4.3. Chemical inactivation of the paternal genome

DNA Inactivation in spermatozoa by chemical agents that selectively damage DNA is an alternative to gynogenesis induction. The interest in this method is because of its possible application in large-scale production of gynogenotes for aquaculture. Unfortunately, all known chemicals inactivating DNA affect other spermatozoa systems and decrease motility of spermatozoa. Tsoi (1969) was the first to induce gynogenesis using dimethyl sulfate (DMS) as a chemical agent that inactivates DNA in rainbow trout (*Oncorhynchus mykiss*) and the peled (*Coregonus peled*) sperm. In this study, no treatment for restoring the ploidy level was applied and the low survival rates could be explained by the presence of haploid larvae. Only 1.3% of peled embryos were hatched, and after insemination treated with 7.7-mM DMS, only 0.16% reached the age of 1 month. The inactivating ability of DMS was proved after the treatment of spermatozoa with high concentrations of DMS by direct counting of chromosomes of the obtained embryonic cells. Although because of the lack of methods to verify paternal inheritance, the gynogenesis induction results in this study are controversial. Later in 1974, Tsoi found that the survival rate of eggs activated by DMS-treated spermatozoa depended on concentration in similar way to those activated with sperm irradiated with different doses of UV light.

The other candidates for substitution of UV-C irradiation are chemicals from psoralens family. The considerable structural similarity in the damage caused by UV-A (360 nm) in the presence of psoralen and UV-C irradiation were found by Cleaver et al. (1985). The effect of this chemical is based on intercalation into DNA and facilitate the formation of specific covalent bonds with pyrimidines after illumination with UV-A light (Cole et al., 1971). McGarry et al. (2009) showed the potential application of the psoralen derivate aminomethyl-4,5',8-trimethylpsoralen (AMT) as a chemical agent that increases the sensitivity of the *Xenopus* sperm to UV-A light. He showed significantly higher efficiency of partial gynogenesis induction (without the ploidy restoration treatment) in *Xenopus* after treatment of sperm with 1-μM AMT followed by UV-A irradiation, whereas irradiation with only AMT or UV-A showed no significant numbers of haploid larvae in progeny. He also showed that the other derivative of

psoralen 8-methoxypsoralen can be a substitute for AMT, which slightly altered the efficiency of DNA inactivation. Various fluorescent DNA dyes have been known for their mutagenic activity (Matselyukh et al., 2005). For instance, ethidium bromide can intercalate into DNA and form covalent bonds under UV-light (Waring, 1965; Cariello et al., 1988). Hiroshi (1965) obtained gynogenotes of medaka (*Oryzias latipes*) after treatment of sperm with toluidine blue followed by UV-A irradiation, although a substantial part of embryos showed abnormalities and delays in development.

1.4.4. Light-dependent DNA repair in spermatozoa

Photoreactivation is a light-dependent enzymatic mechanism that repairs DNA damage. Sperm kept under visible light after DNA inactivation treatment (e.g., UV irradiation) repairs damaged DNA and restores its fertilization ability. This enables cells to intercalate with gynogenesis induction, thus leading to additional variability of results.

The mechanism of photoreactivation is mostly related to the reversion of pyrimidine dimerization (Moan et al., 1989). Exposure of a cell to UV light results in the formation of pyrimidines dimers, as well as cytosine-cytosine or cytosine-thymine dimers to a certain extent. The pyrimidine dimers block DNA replication by polymerase up to {1.1 the sites with the dimer. An enzyme detects and binds to the damaged DNA site and by absorbing energy from the visible light, which activates it, breaks the bonds holding the pyrimidine dimer together, and thus reverses the UV induced dimerization (Woodhead et al., 1979; Applegate et al., 1988). Photoreactivation in general, is a negative effect during gynogenesis induction. Its influence on DNA inactivation treatment can cause failure in gynogenesis induction. Consequently, fertilization of the eggs by photoreactivated spermatozoa may result in a high percentage of embryos with mutations. Therefore, keeping sperm under light can cause decreasing of gynogenotes survival rates and errors in protocol optimization.

Photoreactivation was taken into account during the induction of gynogenesis in sturgeons by several authors, although only little information on the real influence of photoreactivation on gynogenesis is available. The influence of photoreactivation on gynogenesis in sturgeon was investigated by Recoubratsky et al. (2003) who described shift and deformation of typical Hertwig curve after subjecting activated eggs to visible light. Unfortunately, this study did not consider the intensity of illumination and the results were affected by variability of gynogenesis induction, and thus did not quantitatively describe the influence of photoreactivation.

1.5. Treatment for the restoration of diploidy

Numerous authors have evaluated various physical and chemical treatments to restore diploidy of fish haploid zygotes undergoing androgenetic or gynogenetic treatment. In general, this treatment during meiotic gynogenesis is equal to shock used for triploidization (i.e., to prevent extrusion of the second polar body of the egg during the second phase of meiosis), whereas during mitotic gynogenesis or androgenesis, the treatment is equal to shock used for tetraploidization (i.e., to block the first mitotic division). This treatment during the first mitotic division can be used to produce highly homozygotic progeny called double haploids (Ihssen et al., 1990; Pandian & Koteeswaran, 1998; Komen and Thorgaard, 2007). Attempts of mitotic restoration of diploidy have lower success rates and thus have limited usage, such as breeding, genetic research, or the production of tetraploid progeny (Ihssen et al., 1990; Pandian and Koteeswaran 1998). The second polar body of the fertilized egg can be retained without special treatment (e.g., natural gynogenesis, fluctuation of incubation temperature,

or cytoskeletal changes in aging postovulated yet unfertilized eggs), or it may result from temperature, pressure, or chemical treatment of a fertilized egg (Tiwary et al., 2004; Piferrer et al., 2009). These treatments interfere with the normal function of the spindle apparatus, and thus blocking pronuclear movement (Flajshans et al., 2006).

1.5.1. Temperature treatments

These treatments are based on the exposure of recently inseminated eggs to water with a sudden increase/decrease of temperature far beyond its optimum level required for the incubation of a particular species. This is usually done by immersion of the eggs incubated at a normal temperature into hot or cold water for a certain period of time (Flajshans, 2006). For species with sticky eggs, e.g., *cyprinids* or *acipenserids*, eggs can be either desticked prior to exposure to the temperature shock or can be immersed into hot or cold egg-desticking medium. Effective heat shock temperatures are close to the upper limit of tolerance as they act through depolymerization of protein complexes (Komen and Thorgaard, 2007). Cold shock in general has been most successful in warm water fishes (Tiwary et al., 2004; Nakanishi, 1985) and vice versa. Temperature shock treatments are recommended as handy and inexpensive way to restore ploidy. On the other hand, malformations reportedly occur during embryo/larvae development after the application of temperature shock. For restoration of diploidy in sturgeon eggs, they are usually exposed to temperatures ranging from 33 to 37 °C and a duration of 2–5 min at 0.2–0.35 τ_o (where τ_o is duration of the first mitotic cycle; Badrtdinov et al., 2008; Eenennaam et al., 1996b; Fopp-Bayat et al., 2007; Omoto et al., 2005; Recoubratsky et al., 2003; Mims et al., 1997).

1.5.2. Chemical

Chemical ploidy restoration is based on the interaction of chemicals such as colchicine, caffeine, 6-dimethylaminopurine, or cytochalasine B with cells that have a normally functioning spindle apparatus. This method is widely used for the induction of gynogenesis in plants. In fish, it was reported that chemical treatment had a lower success rate than the commonly used temperature or hydrostatic pressure treatments (Bolla & Refstie, 1985). In addition, the use of cytochalasin B and colchicine resulted in the presence of mosaic individuals, e.g., Atlantic salmon, *Salmo salar* (Allen & Stanley, 1979), brook trout, and *Salvelinus fontinalis* (Smith & Lemoine, 1979). Furthermore, the use of triploidization agents such as cytochalasin B is not permitted for aquaculture purposes in European Union countries (Piferrer et al., 2009). Therefore, chemical ploidy restoration has found limited usage in chromosomal manipulations in fish.

1.5.3. Hydrostatic pressure treatments

Hydrostatic pressure shock treatment has been successfully used to block the second polar body extrusion in many commercial species, e.g., rainbow trout (Chourrout, 1984.), common carp *Cyprinus carpio* (Linhart et al., 1991), tench, *Tinca tinca* (Flajšhans et al., 1993), coho salmon *Oncorhynchus kisutch* (Piferrer et al., 1994), summer flounder *Paralichthys dentatus* (Heidi et al., 2009), and hybrid channel catfish *Ictalurus punctatus* × blue catfish *Ictalurus furcatus* (Goudie et al., 1995; Tiwary et al., 2004). This treatment involves exposure to a sudden increase of hydrostatic pressure on recently inseminated eggs. Pressure shock treatment is performed by the transfer of eggs to specially designed pressure chambers where hydrostatic pressure can be increased by a hydraulic press activated either by a pressurized bottled gas

(Linhart et al., 1991) or by an air compressor (Linhart et al., 2001). The optimal amount of pressure shock to prevent the extrusion of the second polar body is similar in most fish species and ranges from 58 to 85 MPa (Piferrer et al., 2009). This treatment requires nonsticky eggs; thus, adhesive demersal eggs (typical for most cyprinids or sturgeons) should undergo desticking procedures prior to the shock treatment.

1.6. Aims of the thesis

The main aims of this study are as follows:

1. To study the key points of optimizing the gynogenesis induction protocol in model sturgeon species.
2. To investigate the ability of chemical substances to inactivate DNA in sturgeon sperm for the induction of large-scale gynogenesis.
3. To estimate the level of light-dependent DNA repair in sperm, and its influence on results of gynogenesis induction.
4. To determine the applicability of interspecific gynogenesis as a method for separating gynogenetic progeny.

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CHAPTER 2

OPTIMIZATION OF SPERM IRRADIATION PROTOCOL FOR INDUCED GYNOGENESIS IN SIBERIAN STURGEON, *ACIPENSER BAERII*

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Optimization of sperm irradiation protocol for induced gynogenesis in Siberian sturgeon, *Acipenser baerii*

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Abstract The great diversity of optimal UV irradiation doses are used for DNA inactivation in fish sperm forcing authors to repeat optimization of irradiation treatment every time. Analysis of sperm UV irradiation protocol for induction of gynogenesis showed the importance of sperm UV light absorption estimations. The UV absorption investigation in Siberian sturgeon sperm showed average extinction coefficient $7.69 \times 10^{-8} \pm 1.04 \times 10^{-8}$ cm^2. It is resulted in high heterogeneity of UV irradiation of undiluted sperm samples. Therefore, it is strongly suggested to specify doses only with defined concentration of spermatozoa; otherwise, the difference in absorbance level between samples can bring a significant error to optimal UV dose estimation. This was confirmed by UV-irradiated sperm motility investigation. Results of motility investigation of UV-irradiated sperm revealed high sensitivity of Siberian sturgeon spermatozoa motion mechanisms to UV irradiation, with complete loss of motility after homogeneous UV irradiation at doses above 2,000 J/m^2. Partial gynogenesis was conducted using diluted and undiluted sperm. Ploidy level of hatched larvae was estimated by flow cytometry. Percentage of haploid hatched larvae revealed sperm DNA inactivation efficiency. The highest percentage of haploid putative gynogenotes 19.67 ± 4.19 % was obtained at UV irradiation dose 200 J/m^2 with sperm diluted to 1:4.

Keywords Sturgeon · Gynogenesis · Sperm · UV absorption · Spermatozoa motility

Introduction

Sturgeons are unique and relict lineage of chondrostean fishes now faced with extinction problem, because of over-fishing, pollution and habitat degradation. As a source of black caviar, the group has experienced decades of intense exploitation fueled by a lucrative

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international caviar market (Billard and Lecointre 2001; Pikitch et al. 2005). One of the ways to protect sturgeons from this influence is to decrease costs for aquaculture production of caviar. For this reason, use of methods of uniparental inheritance such as induced gynogenesis is under the spot light (Keyvanshokooh and Gharaei 2010). It is expected that gynogenotes of some sturgeon species can help to obtain mainly female progeny (Van Eenennaam 1997; Keyvanshokooh and Gharaei 2010), as well as mitotic gynogenotes are of interest because of their high homozygosity, and that may give advantage in genetic and breeding studies. Actually, gynogenesis is process of asexual reproduction, where progeny inherits only maternal DNA and spermatozoa are necessary only for activation of egg development. Method of induced gynogenesis consists of two main procedures: (1) inactivation of DNA in spermatozoa and (2) restoration of diploidy. DNA inactivation can be achieved using different chemical and physical methods (Inssen et al. 1990; Komen and Thorgaard 2007). Commonly used method is to irradiate sperm by UV light in order to damage highly sensitive DNA. The mercury germicidal lamps are used for this purpose as the source of short-wave UV irradiation. Don and Avtalion (1993) who worked on tilapia sperm irradiated by UV proposed a decondensation of spermatozoa chromatin to be the main mechanism damaging the cytoplasmic membrane and nuclear envelope leading to inactivation of male nucleus. Lu and Wu (2005) described few reasons that led to high sensitivity of sperm to UV light such as the lack of sunscreens like mycosporine-like amino acids (Karentz et al. 1997) and a high level of polyunsaturated fatty acids in their cellular and intracellular membranes, making them apt to lipid peroxidation, especially because of limited antioxidant potential due to the small cytoplasmic volume (Aitken et al. 1998).

Generally, the highest mortality of gynogenotes is observed during the first stages of embryogenesis (Van Eenennaam et al. 1996; Omoto et al. 2005; Fopp-Bayat et al. 2007). Probably, the main reason for this is low efficiency of eggs activation by reason of spermatozoa's damage. Due to stochastic influence of irradiation on all volume of a spermatozoon, it damages not only the DNA, but as well the motility system and/or acrosome. As a result, these damages cause the decrease in the eggs activation ability of sperm. Au et al. (2002) showed a highly significant correlation between the fertilization rate and sperm motion parameters for the sea urchin, *Anthocidaris crassispina*.

Li et al. (2000) reported SEM data of irradiated sperm of Japanese scallop (*Patinopecten yessoensis*), showing that short-term exposure of sperm to UV irradiation (254 nm) caused the destruction of acrosome and flagellum. As the dose of UV increased, the damage of acrosome became clearly visible until eventually with doses about 1 kJ/m^2, majority of spermatozoa lost their flagella. Abnormalities in these structures play considerable part in reduction in the activation rates of eggs (Li et al. 2000). Such a huge damage made the spermatozoa immotile. As a result, they could not reach an egg and penetrate an envelope. Thereby, it was necessary to find a compromise between full DNA inactivation and destruction of spermatozoa motility system.

There are a few ways to optimize UV irradiation in sturgeon. The most common way is to induce gynogenesis by sperm irradiated with different dose without ploidy restoration treatment. In this case, all haploid larvae can be determined as putative gynogenotes. Application of ploidy restoration treatment adds another variable to optimization process as well as makes identification of gynogenotes more complicated. The reliable method to check the efficiency of gynogenesis induction with diploidy restoration is microsatellite DNA assay. However, this method is more expensive, laborious and requires samples of parental tissue. Gynogenetic progeny can be easily separated if the hybrids are nonviable or by ploidy level if ova are activated by heterologous sperm with another ploidy level.

Dietrich et al. (2005) demonstrated that comet assay could be used for monitoring the effectiveness of fish sperm DNA inactivation by UV irradiation especially in combination with spermatozoa motility investigation. As the result, this approach allows finding a compromise between complete DNA inactivation and maintenance of sperm motility on acceptable level. The other way to suggest UV dose for DNA inactivation was proposed by Mims and Shelton (1995) in shovelnose sturgeon (*Scaphirhynchus platorynchus*). They suggest that 40 % of the spermatozoa lethal dose will be enough for paternal genome inactivation while saving spermatozoa moving activity. In practice, they used linear regression between sperm transmittance and UV dosage for calculation of optimal UV dose.

Usually 254–260-nm UV-C source is used for DNA inactivation, due to the maximum of absorption by DNA in this region. Use of higher intensity of UV irradiation for shorter duration is more advantageous than long-term irradiation of a sample because of negative effects of thermal influence of light and of a so-called photoreactivation process, i.e., activation of enzymes like photolyase that can remove DNA lesions formed by UV light (Ijiri and Egami 1980; Rajeshwar and Donat-P 2002). Natural mechanism of photoreactivation can significantly decrease amount of DNA damage that will manifest in mutagenesis instead of gynogenesis, especially if sperm after treatment is exposed to light for a long time (Moan and Peak 1989). Therefore, many authors kept both the sperm before activation and the activated eggs in darkness, i.e., from the UV irradiation treatment till the first cleavage division (Ijiri and Egami 1980; Recoubratsky et al. 2003). But in some species, removal of CP dimers by enzymatic photoreactivation was found to be more efficient for mutant reduction than for enhancement of survival (Wade and Trosko 1983).

Optimization of the UV treatment is also complicated due to high optical density of sperm. Published data on the UV irradiation for induced gynogenesis include great diversity of irradiation parameters such as dilution of sperm, intensity of light and differences between species. For example, the published doses of UV irradiation to inactivate DNA in common carp (*Cyprinus carpio*) sperm used in induced gynogenesis ranged from 300 J/m^2 (Cherfas et al. 1994) to 9,000 J/m^2 (Stanley et al. 1976), up to 75,600 J/m^2 (Khan et al. 2000) and to 132,000 J/m^2 (Komen et al. 1991). For sturgeons, two main groups of optimal doses for induced gynogenesis are recently described in the literature (see Table 1). Significant differences among them do not correlate with dilution. Furthermore, the parameter most likely causing the optimal dose diversity in sturgeons is the difference in evolution ploidy levels. Ploidy determines parameters that effect on UV light treatment success such as the amount of DNA in spermatozoa and size of the spermatozoa. However, there is no correlation of spermatozoa's DNA amount with published optimal doses, which may indicate a cumulative influence of many factors like dilution, amount of DNA per spermatozoa, spermatozoa size, packing density of DNA, level of photoreactivation, difference in motility apparatus and sperm quality.

Determination of optimal UV irradiation protocols requires knowledge about UV absorption level in sperm with different concentration of spermatozoa. Additionally knowledge of spermatozoa motility depending on UV irradiation dosage is one of the most important criteria for optimal dose determination. Therefore, the goal of this study was to optimize the UV irradiation protocol in Siberian sturgeon by investigating the UV absorbance of sperm and the motility of irradiated spermatozoa, as well as direct finding of optimal UV irradiation dose by conducting partial gynogenesis.

Table 1 The UV irradiation doses used for sperm inactivation for induced gynogenesis in sturgeons

Species	UV dose (J/m^{-2})	DNA content in haploid cell (pgDNA/nucleus)	Dilution ratio (sperm: medium)	Authors
Group A				
Acipenser stellatus	2,838	2.35	1:9	Saber et al. (2008)
Acipenser schrenckii	2,589	4.0	1:4	Zou et al. (2011)
Acipenser transmontanus	2,160	5.1	1:9	Van Eenennaam et al. (1996)
Bester hybrid	2,100	1.8	1:9	Omoto et al. (2005)
Acipenser brevirostrum	1,200	6.89	1:4	Flynn et al. (2006)
Group B				
Acipenser gueldenstaedtii	297	3.94	1:19	Recoubratsky et al. (2003)
Acipenser baerii	288.75	4.15	1:9	Fopp-Bayat (2010)
Acipenser stellatus	243	2.35	1:19	Recoubratsky et al. (2003)
Acipenser ruthenus	135	1.8	1:19	Recoubratsky et al. (2003)
Bester hybrid	135	1.8	1:9	Fopp-Bayat et al. (2007)
Acipenser baerii	135	4.15	1:9	Fopp-Bayat (2007)

Materials and methods

Fish for experiment

Altogether 15 specimens of Siberian sturgeon, *Acipenser baerii*, males with mean weight of 5 kg were used for sperm motility and the absorption study. The sturgeons originated from the Fischzucht Rhönforelle GmbH & Co. KG fish farm near Gersfeld (Germany). Spermiation was induced by injection of carp pituitary extract at 4 mg/kg 24 h before sperm collection according to Linhart et al. (2000) and Psenicka et al. (2007). Sperm was put on ice during 8 h of transport to the FFPW, Vodňany, Czech Republic, where spermatozoa motility was investigated. Males exhibiting the best (>70 %) motility of spermatozoa were chosen for motility investigation (3 males) and for UV absorption study (5 males). The concentration of spermatozoa in each sample was investigated using a Thoma counting chamber, according to Linhart (1991). Gynogenesis was conducted using Siberian sturgeon broodstock from the Genetic Fisheries Centre in Vodňany, Czech Republic. One female and three males with mean weight of 7 kg were used for gynogenesis. Spermiation of Siberian sturgeon males was induced as was described before. Ovulation of females was induced by hormonal stimulation with intramuscularly injected priming dose of 0.5 mg kg^{-1} b.m. carp pituitary suspension in physiological saline, 42 h before the expected ovulation and 12 h later with the resolving dose of 4.5 mg kg^{-1} b.m. of identical suspension according to Gela et al. (2008). Ovulated eggs from three females were sampled using the microsurgical incision of oviducts as described by Štěch et al. (1999).

UV absorption in sperm

The UV transmission was checked for the determination of the highest effective depth of sperm layer and possible level of UV irradiation heterogeneity. Absorbance levels in sperm

were investigated by spectrophotometer SPECORD 210 in a 1-cm-deep quartz cuvette. Spectrum was measured comparatively to empty cuvette in range 230–310 nm. Several samples were taken for UV transmission from different males with different spermatozoa concentration, and each sample also was measured in dilutions 1:200, 1:100, 1:50, 1:20 and 1:10 by physiological solution. One sample from each male was centrifuged at 5000 RFC for 10 min, after that seminal fluid was collected and absorption level was investigated.

UV irradiation

The UV irradiation of sperm was provided by UV crosslinker CL-1000 (254 nm, Ultra-Violet Products Limited, England), with light intensity 45 W/m^2. For sperm dilution was used artificial medium with ionic concentrations similar to native seminal fluid according to Psenicka et al. (2008). Small amount of sperm (50 µl) with dilutions 1:1, 1:3 or undiluted were poured on watch-glass and distributed at circle with diameter from 1.5 to 2 cm that corresponded with layer depth from 0.16 to 0.28 mm. Watch-glass was kept on ice during irradiation and until analysis. Undiluted sperm were irradiated with doses 2, 5, 7, 7.5 and 10 kJ/m^2. Sperm diluted with ratio 1:1 were irradiated with doses 0.5, 1, 1.5 and 2 kJ/m^2. Sperm diluted with ratio 1:3 were irradiated with doses 0.5, 1 and 2 kJ/m^2. For gynogenesis, 200 µl of diluted sperm was irradiated by UV on Petri dishes (diameter 90 mm) with doses 0.45, 1.35, 2.7, 5.4 and 10.8 kJ/m^2 (nondiluted sperm) and 0.1, 0.2, 0.5, 1, 1.5 and 2 kJ/m^2 (sperm diluted with ratio 1:4). During irradiation, Petri dishes were gently agitated at 50 rpm. After irradiation, 20 ml of water (15 °C) was added to each sample and this suspension immediately added to the eggs. Eggs were fertilized 2 min with gentle stirring at 16 °C, and then distributed into 3 Petri dishes. After sticking of eggs, Petri dishes were immersed into incubators and kept in them at 16 °C until hatching.

Sperm motility and velocity investigations

Spermatozoa motility and velocity were assessed according to method described by Linhart et al. (2000) using SONY SSC DC50AP video camera coupled to Olympus BX-41 microscope with stroboscopic light source (50 Hz). The relationship of mean spermatozoa velocity with time after activation was interpolated, and the relationship of mean spermatozoa velocity at 30 s after activation with irradiation dose was found.

Ploidy estimation

All Hatched larvae of Siberian sturgeon were taken for ploidy level measurement as relative DNA content using Partec Cell Counter Analyser according to Lecommander et al. (1994). Nuclei in separated and permeabilized cells were stained with fluorescent DNA dye, 4′, 6-diamidino-2-phenylindol (DAPI) which easily passed through the nuclear membrane and bounded to base pair sequences in DNA. Not treated larvae of paleotetraploid (4n) sterlet, *Acipenser ruthenus,* were taken as a control group.

Statistics

All values are expressed as mean values ± SD. Data were tested for normal distribution and analyzed by Statistica 9 software, using one-way ANOVA, followed by a Tukey's test for comparisons of mean. The level of significance was set at 0.05.

Results and discussion

UV absorption level in sperm

The sperm under study exhibited very high levels of absorption for UV light, with absorption coefficients close to those in glass. The average absorption coefficient $\alpha \approx 20\ \mathrm{cm}^{-1}$ (with spermatozoa concentration $3 \times 10^8\ \mathrm{ml}^{-1}$, at wavelength 254 nm) means that 1-mm layer of 10-times diluted sperm would absorb from 38 to 39 % of the UV light intensity, and for undiluted sperm, it would be approximately from 98 to 99 % (Fig. 1). According to these data, an extinction coefficient was calculated. Average extinction coefficient for Siberian sturgeon sperm was $7.69 \times 10^{-8} \pm 1.04 \times 10^{-8}\ \mathrm{cm}^2$. However, these calculations were valid only for diluted sperm, because of high absorption as well as because the undiluted sperm was highly scattering fluid (Khandpur 2007; Fauvel et al. 2010). Based on these results, it is strongly suggested to specify doses and dilutions only with defined concentration of spermatozoa; otherwise, the difference in absorbance level between samples can bring a significant error.

Motility of UV-irradiated sperm

Diluted samples showed quick continuous exponential decrease in mean velocity of spermatozoa with increase in UV dose (Fig. 2). These results could be explained by previously described damages of spermatozoa motility apparatus in the other species (Li et al. 2000; Don and Avtalion 1993; Dietrich et al. 2005). A slow decrease in spermatozoa motility with increasing UV dose was found in undiluted sperm. Slowdown of changes was probably caused by differences in irradiation level in the upper and bottom layers of sperm of the undiluted sample. Therefore, even highly irradiated sperm sample contained spermatozoa from bottom layer that received low doses of UV. The percentage of motile

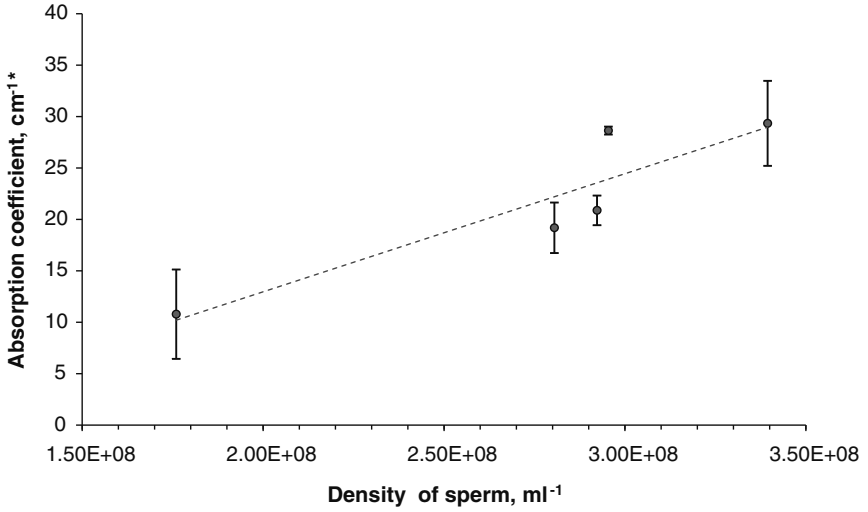

Fig. 1 Absorption coefficients in sperm of five Siberian sturgeons with different spermatozoa concentrations, at 254 nm ($R^2 = 0.83$)

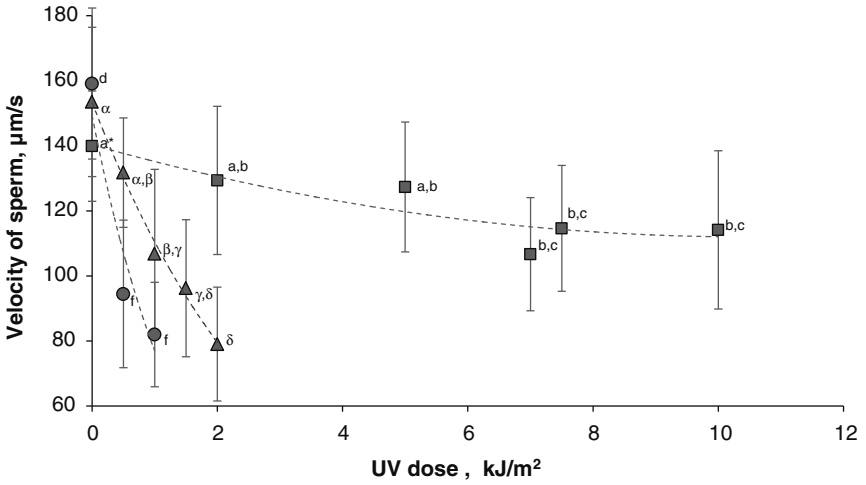

Fig. 2 Motility of UV-irradiated sperm as a function of dose, with different dilutions at 30 s after activation, *filled square* sperm without dilution, *filled triangle* sperm with dilution 1:1. *Filled circle* sperm with dilution 1:3. Spermatozoa concentration in undiluted sperm is 2.95×10^8 ml^{-1}. **a*, *b*, *c*; α, *β*, *γ*, *δ*; *d*, *f* statistically different groups, $p < 0.05$ (ANOVA, Tukey's test)

spermatozoa showed similar high sensitivity to UV irradiation and the influence of shadowing (Fig. 3). These results are in accordance with the results of Li et al. (2000) working with Japanese scallop sperm, who showed the percentage of spermatozoa that lost their flagella under UV irradiation, and Porter (1998), who irradiate sperm of bluegill (*Lepomis macrochirus*). Without dilution, small percentage of spermatozoa saved their motility even if exposed to high UV doses (up to 10 kJ/m^2, Fig. 3), and this fact could contribute to explain the previous results obtained by authors who have used high doses for sperm inactivation like in large yellow croaker (*Pseudosciaena crocea*) 750,000 J/m^2 (Jian-He et al. 2007), common carp 75,600 J/m^2 (Khan et al. 2000), winter flounder (*Pseudopleuronectes americanus*) 36,000 J/m^2 (Hornbeek and Burke 1981), Wuchang bream (*Megalobrama amblycephala*) 36,000 J/m^2 (Luo et al. 2011) and rainbow trout (*Oncorhynchus mykiss*) 33,000 J/m^2 (Colihueque et al. 1992).

Induction of gynogenesis

Investigation of ploidy in larvae incubated from eggs that were activated by undiluted UV-irradiated sperm has shown high percentage of diploid no gynogenetic larvae (Table 2).

The haploid as well as diploid larvae were obtained from sperm irradiated with very high doses up to 10 kJ/m^2. These results indicate a significant heterogeneity of UV irradiation doses obtained by spermatozoa in the undiluted sperm sample and correlate with sperm motility investigation described above. On the other hand, use of sperm diluted by no activated medium before UV irradiation showed low percentage of diploid larvae in progeny and higher hatching rate.

The highest percentage of hatched gynogenotes was obtained with UV irradiation dose from 100 to 200 J/m^2 providing that spermatozoa were homogeneously irradiated. These are the points to the validity of the results of authors who proposed relatively low optimal doses of UV light (see Table 1) Recoubratsky et al. (2003) and Fopp-Bayat (2010). Both

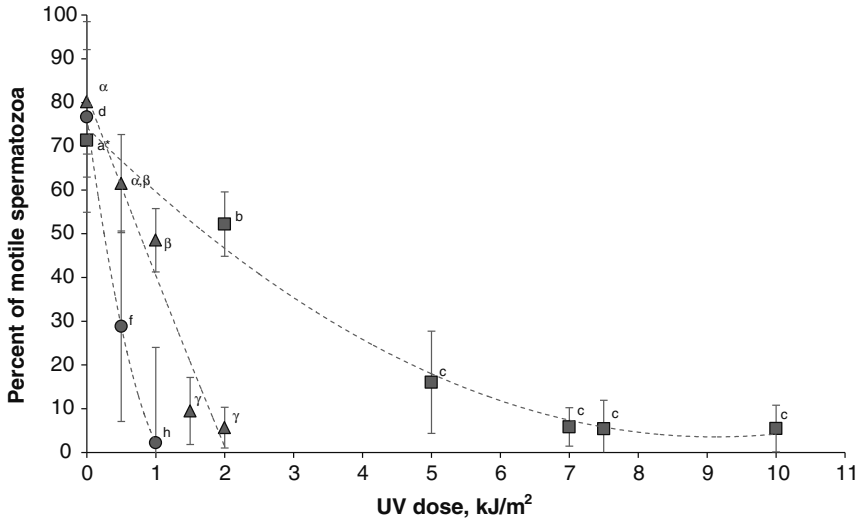

Fig. 3 Percentage of motile spermatozoa in UV-irradiated sperm with different dilution rates at 30 s after activation. *Filled square* sperm without dilution, *filled triangle* sperm with dilution 1:1, *filled circle* sperm with dilution 1:3. Spermatozoa concentration is 2.95×10^8 ml^{-1}. **a*, *b*, *c*; *α*, *β*, *γ*; *d*, *f*, *h* statistically different groups, $p < 0.05$ (ANOVA, Tukey's test)

Table 2 Hatching rate and percentage of diploid larvae after fertilization by irradiated sperm of Siberian sturgeon

Dilution ratio	Dose (J/m^2)	Number of eggs	Hatched larvae (% ± SD[1])	Diploid larvae (% ± SD)
1:0	450	376	$6.98 \pm 0.65^{a,**}$	5.94 ± 0.76^{e}
	1,350	322	6.51 ± 1.24^{a}	2.18 ± 0.44^{f}
	2,700	354	13.14 ± 3.8^{b}	5.98 ± 2.15^{f}
	5,400	350	5.76 ± 0.53^{c}	1.38 ± 0.58^{g}
	10,800	333	2.33 ± 1.32^{d}	0^{h}
1:4	0	419	87.59 ± 7.97^{k}	87.59 ± 7.97^{q}
	100	190	17.54 ± 1.58^{l}	1.59 ± 0.08^{r}
	200	422	19.91 ± 4.18^{l}	0.24 ± 0.01^{s}
	500	417	13.19 ± 2.11^{m}	0.24 ± 0.01^{s}
	1,000	416	10.58 ± 3.07^{m}	0
	1,500	421	2.14 ± 1.71^{n}	0
	2,000	426	0^{p}	0

** a, b, c, d; e, f, g, h; k, l, m, n, p; q, r, s—statistically different groups, $p < 0.05$ (ANOVA, Tukey's test)

[1] Percentage relative to initial number of eggs ± standard deviation

these authors use high rates of sperm dilution. As a result, sperm was irradiated homogeneously and low UV doses were sufficient to inactivate DNA. It can be explained by previously reported reasons that led to high sensitivity of sperm to UV, like a lack of sunscreens, a high level of polyunsaturated fatty acids, limited antioxidant potential and high level of the spermatozoa DNA compaction (Karentz et al. 1997; Aitken et al. 1998; Lu and Wu 2005).

Conclusion

The UV-irradiated sperm motility investigation showed high sensitivity of spermatozoa motility system. Furthermore, it showed strong dependence of irradiation heterogeneity of sample on its dilution ratio that is explained by the investigation of the UV light absorption level in sperm. The described relationship of the absorption coefficient with the concentration of sperm allows an estimation of the level of absorption in the sample based on the number of spermatozoa per volume unit. This information can be useful for compiling protocols of induced gynogenesis in sturgeons. Investigation of gynogenesis efficiency confirmed high importance of dilution and showed optimal UV doses for gynogenesis induction below 200 J/m^2 for homogenously irradiated sperm.

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CHAPTER 3

CHEMICAL INDUCTION OF HAPLOID GYNOGENESIS IN STERLET, *ACIPENSER RUTHENUS*

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Chemical induction of haploid gynogenesis in sterlet *Acipenser ruthenus*

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ABSTRACT: Chromosomal manipulations in sturgeons, particularly gynogenesis, are interesting due to the potential to change female ratio in progeny that can be useful for caviar production. The optimization of UV treatment for induction of gynogenesis is complicated due to high and variable optical density of the milt due to differential spermatozoa concentration, and because of sensitivity of spermatozoa's motility apparatus. Therefore in this study we compared chemical methods of sperm treatment as an alternative to short wave-length UV treatment; evaluation considers impact on spermatozoa motility, DNA integrity, and efficiency of DNA inactivation. Dimethyl sulfate (DMS) in concentrations of 2.5–30mM was applied to spermatozoa in order to inactivate DNA. Also ethidium bromide (EB), psoralen (PS), and 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) were used to increase sensitivity of spermatozoa's DNA to long wavelength UV-A light (360 nm). CASA analyses of treated sperm showed strong negative effects on spermatozoa motility with the increasing concentration of active substances. Additionally in case of PS, EB, and DMS treatment comet assay did not reveal significant DNA damage of sperm at the range of concentrations relatively safe for spermatozoa motility. Flow cytometric analysis of relative DNA content in larvae resulting from activation of normal ova of sterlet with the treated sperm showed low efficiency of haploid gynogenesis induction. The putative gynogenetic larvae were found after treatment with PS in concentrations higher than 18μM and EB higher than 10μM followed by UV-A irradiation at the dose of 900 J/m^2 and DMS up to 5mM. Because of an overwhelming impact on the sperm motility and relatively low DNA damage, treatment of sperm with PS, EB or DMS did not prove efficient compared with a widely used UV-C irradiation treatment. In contrast, treatment with AMT followed by UV-A showed lower influence on spermatozoa motility and higher efficiency of DNA damaging resulting in the higher percentage of gynogenotes in the progeny, thus could be considered as a possible substitution for UV-C treatment.

Keywords*:* sturgeons; chromosomal manipulation; dimethyl sulfate; psoralen; comet assay

INTRODUCTION

Sturgeons are one of the oldest fish families including the genera *Acipenser*, *Huso*, *Scaphirhynchus,* and *Pseudoscaphirhynchus*. The number of research and developmental studies on sturgeon biology and biotechnology is constantly increasing because their survival in the wild is seriously endangered (Fontana et al. 2001) and they are highly prized for their caviar and flesh. The worldwide population of sturgeons is constantly declining due to over-fishing, water pollution, and habitat degradation (Billard and Lecointre 2001). It is anticipated that the future of sturgeon caviar and flesh production is in developing the aquaculture biotechnologies. Thus it is highly demanding to elaborate methods of sex identification and manipulation with progeny sex ratio. Chromosomal

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manipulation methods, particularly gynogenesis, can be used for changing the female ratio in sturgeon progeny. Despite quite contradictory results of sturgeon sex determination system assessment, female heterogamety is commonly accepted (Keyvanshookoh and Gharaei 2010). In accordance with this hypothesis, high percentage of females in gynogenetic progeny was shown in a number of sturgeon species: *Acipenser transmontanus* 82% (Van Eenennaam 1999), *Acipenser baerii* 81% (Fopp-Bayat 2010), *Polyodon spathula* 80% (Shelton and Mims 2012), bester 70–80% (Omoto et al. 2005), *Acipenser brevirostrum* 65% (Flynn et al. 2006). Furthermore, the other applications of gynogenesis are related to the interesting ploidy level system of sturgeon and its evolution. Due to high homozygosity of gynogenetic progeny, it can be useful in genetic research and selection.

The processes of gynogenesis induction consist of two main steps – the inactivation of paternal DNA in sperm and the restoration of zygote's diploidy. Inactivation of genetic information in spermatozoa for activation of eggs is a critical step for gynogenetic induction protocol. The second step is quite similar to a triploidization method and represents an application of a shock to induce retention of the 2nd meiotic polar body (Fopp-Bayat et al. 2007). The most common way to inactivate DNA in spermatozoa is to irradiate them with UV, but a study on the influence of UV irradiation on motility of sturgeon spermatozoa showed high sensitivity of the motility apparatus to UV light (Recoubratsky et al. 2003; Dietrich et al. 2005). Furthermore, optimization of the UV treatment is complicated due to high optical density of sperm and significant difference in sperm density among individual males (Christensen and Tiersch 1994; Mims and Shelton 1998; Linhart et al. 2000).

The other way to induce gynogenesis is inactivation of DNA in spermatozoa by chemical agent that selectively damages DNA and provides minimum damage to spermatozoa's motility apparatus and acrosome. Tsoi (1969, 1974) used dimethyl sulfate (DMS) as chemical agent inactivating rainbow trout (*Oncorhynchus mykiss*) and peled (*Coregonus peled*) spermatozoa's chromosomal apparatus. He found similarity of survival rate dependence in eggs activated by DMS and UV treated spermatozoa. Increasing of concentrations above the lethal dose has led to the appearance of normally looking larvae. Inactivating ability of DMS was proved, after spermatozoa treating with high concentrations of DMS, by direct counting of chromosomes in obtained embryonic cells. Though eggs of peled showed quite low survival rate after insemination by sperm treated with DMS in the concentration of 7.7mM. Only 1.3% of embryos were hatched and only 0.16% of them reached the age of one month.

Various fluorescent DNA dyes have mutagenic activity (Matselyukh et al. 2005). For instance ethidium bromide (EB) can intercalate to DNA structure, and form covalent bonds under UV-light (Waring 1965; Cariello et al. 1988). Uwa (1965) obtained gynogenotes of medaka (*Oryzias latipes*) after treatment of sperm with toluidine blue followed by UV irradiation, although substantial part of embryos showed abnormalities and delay in development.

A widely investigated family of substances – psoralens – have been known in medicine for many years as amplifiers of DNA sensitivity to UV-A. They can intercalate into DNA structure and form specific covalent bonds with pyrimidines after illumination with 360 nm light (Cole 1971). McGarry et al. (2009) used aminomethyl-4,5',8-trimethylpsoralen (AMT) as a chemical agent that increases sensitivity of *Xenopus* sperm to long wavelength UV light. He showed significantly higher efficiency of enucleation after treatment of sperm with 1μM AMT. Influence of psoralen (PS) on sensitivity of DNA in cells to UV light has long been known and used for skin disease treatment. Cleaver et al. (1985) investigated processes of DNA damage caused by UV-A (360 nm) in presence of PS. He found considerable structural similarity of DNA damage caused by UV-C (254 nm) light and PS treatment followed by UV-A irradiation.

The aim of the present study was to compare different methods of spermatozoa DNA deactivation as an improvement to UV-C treatment which has been the conventional procedure for gynogenetic induction.

MATERIAL AND METHODS

Fish. The experiment was conducted at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Spermiation of 15 sterlet males with average weight of 2 kg was induced by injection of carp pituitary extract at 4 mg/kg of body weight 24 h before sperm collection according to Linhart et al. (2000) and Psenicka et al. (2007). Sperm was collected to 20 ml syringes and put on ice until processing.

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Sperm with potent spermatozoa motility higher than 70% was chosen for further research. Ovulation of 8 sterlet females (average weight 2.5 kg) was induced by hormonal stimulation with intramuscular injection of carp pituitary suspension in physiological saline solution (0.5 mg per kg body weight) 42 h before the expected ovulation, and 12 h later with the resolving dose (4.5 mg per kg body weight) of identical suspension according to Gela et al. (2008). Ovulated eggs were sampled using the microsurgical incision of oviducts as described by Stech et al. (1999).

Dimethyl sulfate treatment. Dimethyl sulfate (DMS) in concentrations 0.5–15mM was applied to sterlet sperm in order to inactivate DNA. The sperm samples (200 µl) were added to 200 µl of DMS solution with concentrations of 1–30mM. Immediately after addition of DMS, the samples were centrifuged at 200 *g* for 6 min. After centrifugation the supernatant was replaced by non-activating solution that has similar ionic concentrations to native seminal fluid (Psenicka et al. 2008) and the centrifugation was repeated. After the second centrifugation the supernatant was replaced similarly and the samples were stored on ice until fertilization assays.

EB, PS, and AMT treatment. PS, AMT, and EB were applied to increase sensitivity of spermatozoa to longwave UV-A (360 nm). 200 µl of PS solutions in concentrations 1, 2.5, 5, 18, 32, and 200µM, EB in concentrations 1, 5, 10, 20, and 100µM, or AMT in concentrations 0, 1, 5, 10, 25, 50, and 100µM were added to the sperm sample (200 µl) and stored at 4°C. After 2–5 min of incubation the samples were poured on Petri dish with 50 mm diameter that corresponds to approximate sample depth of 0.2 mm. Then the samples were irradiated by UV-A light (360 nm) for 180 s at 5 W/m^2 (900 J/m^2). Intensity of irradiation was measured by Black-Ray UV meter J-221 (Ultra-Violet Products Ltd., Cambridge, UK). UV-C (253 nm) irradiation was conducted by UV crosslinker CL-1000 (Ultra-Violet Products Ltd.), with light intensity 45 W/m^2. Irradiated samples were transferred to Eppendorf tubes covered with foil and kept on ice.

Table 1. Curvilinear velocity of motile spermatozoa after treatment with DMS, AMT, PS or EB 30 s after activation

Chemicals	Concentration	UV-A (360 nm), J/m^2	Average curvilinear velocity ± SD (µm/s)
–	0	0	188.8 ± 9.6
–	0	900	158.1 ± 8.3
PS	10µM	900	144.2 ± 10.1
PS	18µM	900	136.9 ± 11.7
PS	32µM	900	120.4 ± 11.5
PS	50µM	900	0.0
DMS	25mM	0	168.4 ± 8.7
DMS	30mM	0	164.4 ± 10.4
DMS	35mM	0	162.1 ± 6.5
DMS	50mM	0	0.0
EB	20µM	900	140.0 ± 9.0
EB	50µM	900	132.2 ± 6.8
EB	100µM	900	129.6 ± 5.2
EB	200µM	900	0.0
AMT	0µM	900	167.4 ± 34.3
AMT	0.5µM	900	170.6 ± 40.9
AMT	1µM	900	162.3 ± 44.9
AMT	5µM	900	178.0 ± 26.2
AMT	10µM	900	171.5 ± 24.2
AMT	25µM	900	173.3 ± 24.2
AMT	50µM	900	153.4 ± 26.8
AMT	100µM	900	128.6 ± 22.1

DMS = dimethyl sulfate, AMT = 4'-aminomethyl-4,5',8-trimethylpsoralen, PS = psoralen, EB = ethidium bromide, SD = standard deviation

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Sperm motility and velocity investigations. Spermatozoa motility was triggered by dilution with non-chlorinated tap water at 1 : 50. Spermatozoa motility and velocity were assessed according to Linhart et al. (2000) using SONY SSC DC50AP video camera (SONY, Tokyo, Japan) coupled to Olympus BX-41 microscope (Olympus Corp., Tokyo, Japan) with stroboscopic light source (50 Hz). Velocity and motility were assessed at 30 s post-activation. The successive positions of the video recorded spermatozoa heads were analyzed from five video frames using Olympus MicroImage software (Version 4.0.1., 1999, for MS Windows with a special macro by Olympus C & S). 20–40 spermatozoa were counted per each frame. Spermatozoa velocity (μm/s) was calculated based on length of spermatozoa traces, calibrated for magnification.

Comet assay. Sperm suspension after treatment was diluted with pre-chilled PBS 1 : 250 and stored at 4°C. Agarose gel (0.8% NuSieve GTG low melting point agarose OxiSelect™; Cell Biolabs, Inc., San Diego, USA) was melted at 90°C and chilled to 37–40°C. Agarose gel was mixed with diluted sperm at the ratio 9 : 1. Microscope slides (OxiSelect™; Cell Biolabs, Inc.) were used for the assay. Immediately after mixing, 50 μl of suspension was pipetted to each well on the slide and spread over the well. Slides were stored at 4°C until solidification of the agarose gel, and then immersed in lysis solution prepared according to the OxiSelect Comet Assay protocol with addition of proteinase K (1 mg/ml of lysis solution). Slides were incubated in lysis solution for 10 h at 37°C. After incubation, lysis solution was carefully aspirated and replaced by pre-chilled electrophoresis solution (90mM Tris Base, 90mM boric acid, 2.5mM EDTA, pH 8.0). After 5 min the slides were carefully transferred to electrophoresis chamber with cold electrophoresis solution. Electrophoresis was carried out at 1 V/cm for 20 min, and then slides were transferred for 5 min to a small container with pre-chilled distilled water. Distilled water was carefully aspirated and slides were air-dried. Once the agarose slide was completely dry, 20 μl per well of Vista Green DNA Staining Solution (OxiSelect™; Cell Biolabs, Inc.) were added. Slides were viewed under Olympus Fluoview microscope (Olympus Corp., Tokyo, Japan) using filters with 450–480 nm excitation wavelengths and recorded by SONY DXC-9100D videocamera (SONY, Tokyo, Japan). The DNA damage in spermatozoa was evaluated using CASP freeware (Version 1.2.3., 2001).

Fertilization and hatching rate. To verify the DNA inactivation for perspective application in gynogenesis induction, solutions with 200 μl of sperm of each sample were added to 20 ml of water from the incubation system and immediately mixed with 5 g of eggs (350–375 eggs). Eggs were activated during 2 min with gentle stirring at 16°C, and then distributed into 3 Petri dishes. After sticking the eggs to the bottom, Petri dishes were immersed into hatching trays in an incubation system (Linhart et al. 2003) and kept at 16°C until hatching. No gynogenetic induction was done, evaluation was based on production of haploid larvae. Hatching rates were estimated as percentage of hatched larvae relative to the amount of eggs used for fertilization.

Ploidy level estimation. Hatched larvae were taken to verify the haploid level. Ploidy was estimated using Partec CCA flow cytometer (Partec GmbH, Münster, Germany) according to Lecommander et al. (1994). Caudal part of each larva was used for determination of relative DNA content per cell. The untreated *Acipenser ruthenus* larvae with ploidy level equal to evolutionary tetraploid (Havelka et al. 2011) were taken as a control group. Separated cells were stained with fluorescent DNA dye, DAPI (4', 6- diamidino-2-phenylindol) which easily passed through the nuclear membrane and bound to base pair sequences in DNA.

Statistical analysis. All values were expressed as mean values ± standard deviation (SD). Data were tested for normal distribution and analyzed by STATISTICA software (Version 9.0, 2009), using One-Way ANOVA, followed by a Tukey's test for comparisons of means. The level of significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

The strong influence of DMS and PS on the motility parameters of spermatozoa was found at concentrations significantly affecting the DNA integrity (Table 1, Figures 1, 3, 4).

Treatment with EB or AMT followed by UV-A irradiation showed slower decrease of spermatozoa motility with increasing concentrations compared to other substances. Namely UV-A irradiation led to significant decrease of motility in the case of AMT, EB, and PS.

Although DMS concentrations of 25–35mM affected spermatozoa motility and velocity less than the treatment with EB or PS followed by UV-A irradiation, presence of 50mM of DMS totally

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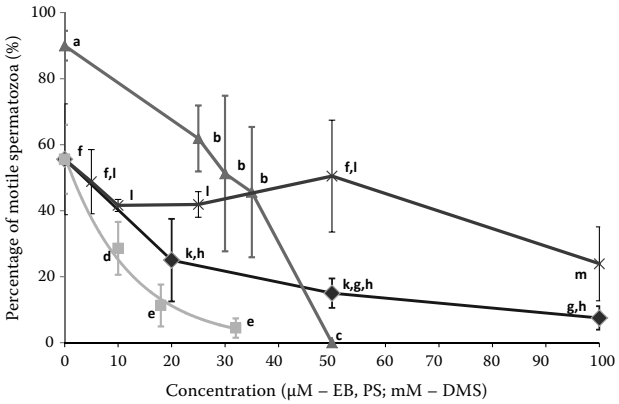

Figure 1. Percentage of motile spermatozoa at 30 s after activation

sperm treated with DMS (▲); PS followed by UV-A (■); AMT followed by UV-A (×); EB followed by UV-A (♦)

DMS = dimethyl sulfate, PS = psoralen, AMT = 4'-aminomethyl-4,5',8-trimethylpsoralen, EB = ethidium bromide

$^{a–h, k–m}$statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

inhibited spermatozoa motility which makes the application of this treatment limited.

As a next step in the assessment of the perspectives of application of AMT, DMS, EB, PS, and UV irradiation for DNA inactivation, the influence of these factors on DNA integrity was evaluated (Figures 2, 3). The highest level of DNA fragmentation was achieved by treatment with DMS at concentration of 100mM, but as seen in Figure 1, motility of spermatozoa was totally inhibited already at 50mM of DMS. Nevertheless, treatment with DMS at concentrations 25–35mM did not completely inhibit motility and significantly increased the level of DNA damage compared to control. Longwave UV-A irradiation (360 nm, 900 J/m^2) did not cause significant damage to the DNA of sperm cells (Figure 3B, C) in contrast to UV-C (260 nm) irradiation, where the same irradiation dose caused 5 times higher DNA damage compared to control (Figure 3D). This correlates with the dependence of the level of pyrimidine dimer formation on wavelenghts of UV-light discribed by Enninga et al. (1986).

Additionally it was shown that damage caused by AMT + UV-A, PS + UV-A, and EB + UV-A treatments did not originate only from UV-A irradiation or the substances, but actually it was caused by the combination of each substance with UV-A irradiation. However, despite the lower sensitivity of DNA to UV-A light, motility apparatus retained highly sensitive to UV-A. Therefore, although the addition of AMT, EB, and PS caused increase of the DNA sensitivity to the longwave UV-A light (Figures 2, 3B, C), it also negatively influenced sperm motility (Figure 1), which did not allow to obtain reasonable fertilization leading to higher survival rates (Figure 4A, B). On the other hand, lower influence of AMT treatment on motility systems and higher DNA damage resulted in higher percentage of gynogenotes in progeny (Figure 5).

Flow cytometric analysis of larvae hatched from the eggs activated by DMS treated spermatozoa did not show any haploid larvae up to the dose of 5mM DMS; the observation is consistent with the result of gynogenesis induction in peled and rain-

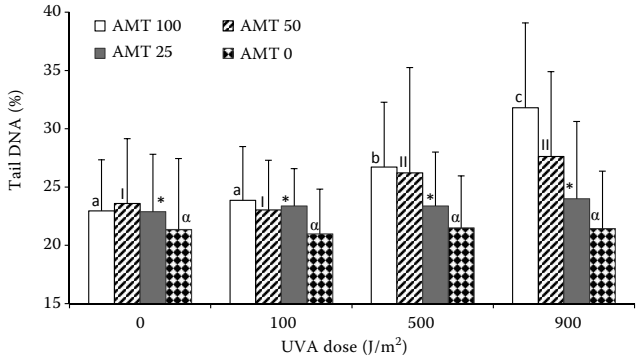

Figure 2. Level of DNA damage in spermatozoa measured by comet assay after treatment with 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) (0, 25, 50, 100μM) followed by UV-A (0, 100, 500, 900 J/m^2)

$^{a–c;\ I–II;\ *;\ \alpha}$statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

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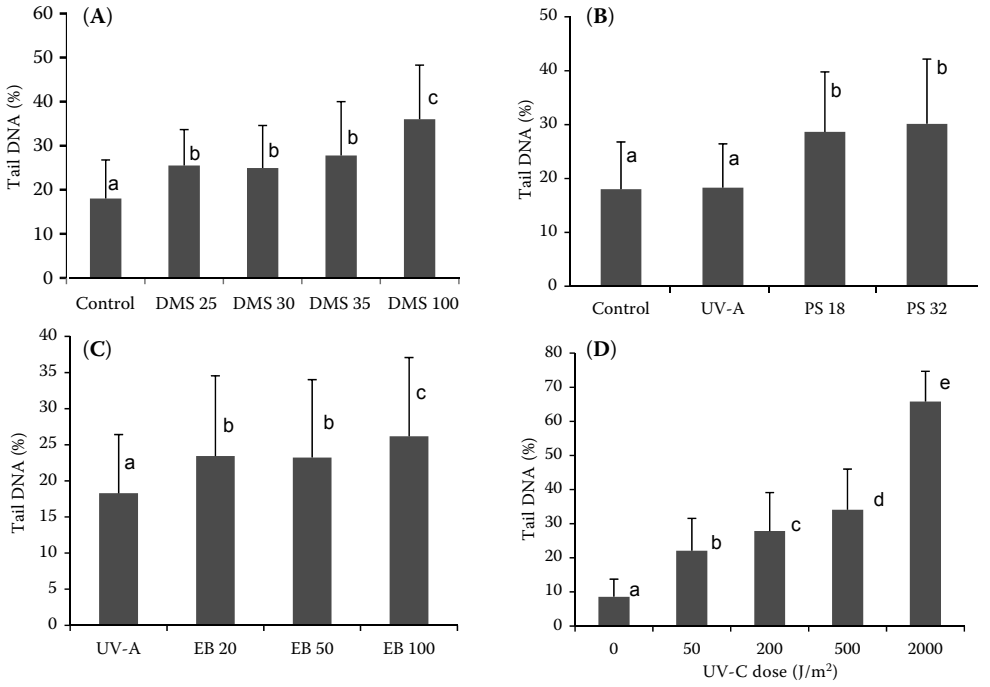

Figure 3. Level of DNA damage in spermatozoa measured by comet assay after treatment with DMS (25, 30, 35, 100mM) (**A**); PS (18, 32μM) + UV-A (900 J/m^2) and UV control (UV-A irradiation 900 J/m^2) (**B**); EB (20, 50, 100μM) + UV-A (900 J/m^2) (**C**); UV-C (254 nm) (**D**)

DMS = dimethyl sulfate, PS = psoralen, EB = ethidium bromide

$^{a-e}$statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

bow trout by Tsoi (1969). We suppose that such a low survival rate in the experiment of Tsoi could be explained by the lack of the method on separating spermatozoa from DMS solution after treatment and preventing the direct effect of DMS on eggs.

Purely haploid progeny was obtained after treatment with doses of about 30mM of DMS (Figure 4B) but only few spermatozoa were motile at this concentration. This was the reason to assume that DMS did not damage DNA sufficiently for prospective induction of gynogenesis at concentrations safe for sperm motility apparatus.

Similarly to DMS, ploidy investigations of larvae developed from sperm treated with PS at doses up to 18μM and EB up to 10μM and followed by UV-A irradiation at the dose of 900 J/m^2 did not reveal any significant amount of haploid larvae (Figure 4B). That is consistent with our result of comet assay and sperm motility investigation. In general, the amount of hatched larvae correlated with sperm motility analysis (Figure 4A) and showed strong negative correlation between the doses of chemical agents and hatching rate.

The highest percentage of putative haploid gynogenetic larvae (up to 19.81%) was obtained after treatment of sperm with AMT at the dose of 100μM followed by UV-A irradiation at 900 J/m^2. Further optimizations of AMT treatment led us to possible substitution of UV-irradiation method by the chemical treatment. However, chemical treatment of spermatozoa may impact on the progeny development, therefore further investigation is required.

CONCLUSION

All tested chemicals can be used for induction of gynogenesis in sterlet. Nevertheless DMS showed low efficiency of DNA inactivation and selectiveness of the influence on sterlet sperm. The DMS concentrations completely inactivating sperm DNA

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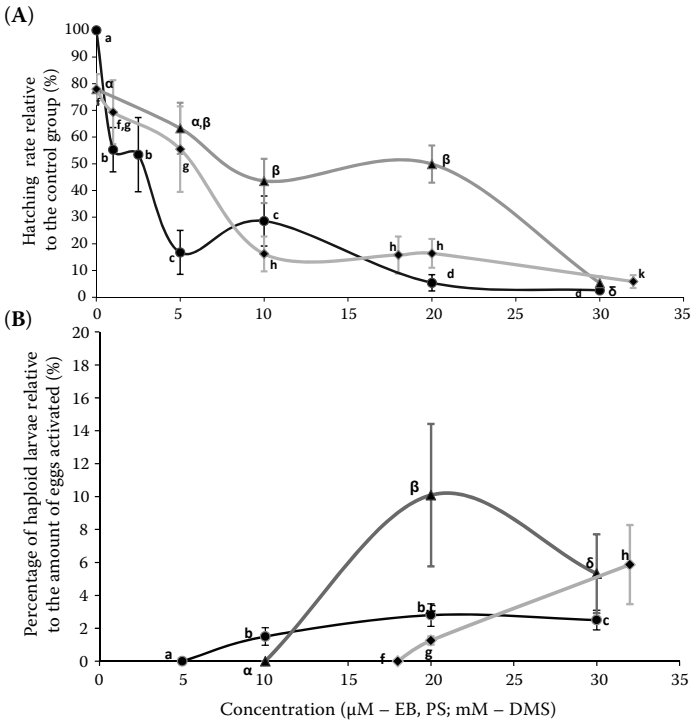

Figure 4. Hatching rate (relative to the survival of control group) (**A**) and percentage of putative gynogenetic larvae (relative to the amount of eggs) (**B**) after treatment of sperm with DMS (●); PS + UV-A (900 J/m^2) (♦); EB + UV-A (900 J/m^2) (▲)

DMS = dimethyl sulfate, PS = psoralen, EB = ethidium bromide

superscript letters indicate significant differences between means (ANOVA, $P < 0.05$)

greatly reduced the number of motile spermatozoa thereby creating a limit to the DMS effectiveness at about 2–3% of the control hatching rate. Treatment with PS and EB also showed a strong overwhelming impact on the sperm motility. At the same time, compared with a widely used UV-C irradiation treatment, it did not prove to be more efficient. On the other hand, treatment with AMT showed quite low influence on sperm motility and as a result could be used as substitution for UV-C

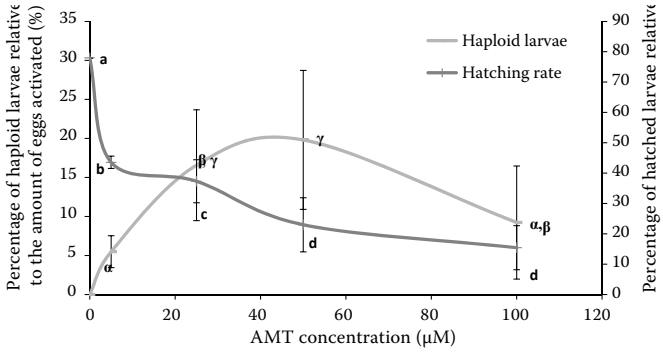

Figure 5. Hatching rate and percentage of haploid larvae in progeny obtained from sperm treated with 4'-aminomethyl-4,5'8-trimethylpsoralen (AMT) in concentrations of 0, 5, 25, 50, 100μM followed by UV-A irradiation (900 J/m^2)

$^{a–d, \alpha–\gamma}$statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

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irradiation. Despite the obscure results of chemical induction of gynogenesis, the finding of new ways of DNA inactivation could help us substitute the difficultly optimizable UV-irradiation method by the chemical treatment.

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CHAPTER 4

INFLUENCE OF PHOTOREACTIVATION ON GYNOGENESIS INDUCTION IN STERLET, *ACIPENSER RUTHENUS*

Lebeda, I., Flajšhans, M., 2014. Influence of photoreactivation on gynogenesis induction in sterlet, *Acipenser ruthenus*. (manuscript)

INFLUENCE OF PHOTOREACTIVATION ON INDUCTION OF GYNOGENESIS IN STERLET, *ACIPENSER RUTHENUS*

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Abstract

Chromosomal manipulations in sturgeons, particularly gynogenesis, can be used to alter the female progeny ratio and thus be useful for caviar production. The most common method to inactivate spermatozoa DNA for gynogenesis is UV-C irradiation. Because of the presence of light-dependent DNA repair systems, changes in DNA damage under light can cause substantial errors in determining optimal UV-dose. DNA reactivation of UV-irradiated sperm, as measured by the comet assay, resulted in a significant level of DNA restoration in sperm maintained under artificial light conditions. The induction of gynogenesis demonstrated significantly different success rates after the UV-C irradiated sperm was subjected to incandescent bulb light (1350 Lm, 50 cm) for 0, 5, and 10 min. The level of DNA restoration was higher in sperm irradiated with higher doses; thus, the influence of reactivation was particularly significant when higher doses of UV-C were applied. In addition, effects of infrared and long wavelength part of the visible spectrum (600–2000 nm) on UV-irradiated sperm were investigated, and results indicated that the infrared light did not induce light-dependent DNA reactivation. Therefore, using infrared light to illuminate the work place during induction of gynogenesis is suggested.

Keywords: *photoreactivation, gynogenesis, sturgeon, comet assay*

1. Introduction

Sturgeons are faced with extinction because of pollution, habitat loss, and over-fishing. Therefore, the demand for captive breeding and aquaculture of sturgeons to produce caviar has increased, as wild stocks have become depleted or closed for legal harvest. As a result, methods of uniparental inheritance, such as induced gynogenesis, are gaining attention (Keyvanshokooh and Gharaei, 2010). It is expected that gynogenesis can help increase the productivity of sturgeon aquaculture (Van Eenennaam, 1997; Keyvanshokooh and Gharaei, 2010). In addition, mitotic gynogenesis could be interesting because of the high homozygosity of progeny that provides advantages in genetic and breeding studies. In general, the highest mortality of gynogenotes is observed during the first stages of embryogenesis (Van Eenennaam et al., 1996; Omoto et al., 2005; Fopp-Bayat et al., 2007), probably because of the low efficiency of egg activation caused by spermatozoa damage. This stochastic UV damage influences spermatozoa volume, including the motility system and/or acrosome. As a result, this damage causes decrease in eggs activation ability of sperm. Therefore, it is necessary to identify a compromise between full DNA inactivation and destruction of the spermatozoa motility system. Inactivation of sperm genetic information is usually conducted by UV-irradiation at wavelengths of 250–260 nm. However, optimizing this treatment is complex

because of the sensitivity of the spermatozoa motility system, the high optical density of sperm, and the significant difference in sperm density among males. In addition, optimizing an irradiation treatment may be affected by the negative effects of the photoreactivation process, i.e., the activation of enzymes such as photolyase, which can remove DNA lesions formed by exposure to UV light (Ijiri and Egami, 1980; Sinha and Häder, 2002). During normal fertilization, removal of cyclobutane pyrimidine dimers by enzymatic photoreactivation efficiently reduces mutation rate (Wade and Trosko, 1983). In the case of gynogenesis, the naturally occurring mechanism of photoreactivation can significantly decrease the amount of DNA damage that manifests in mutagenesis instead of gynogenesis, particularly if sperms are exposed to light for a long duration after treatment (Moan and Peak, 1989). Therefore, many previous studies kept sperms and activated eggs in the dark before activation, i.e., away from UV irradiation until the first cleavage division (Ijiri & Egami, 1980; Recoubratsky et al., 2003).

The main goal of this study was to investigate the level of sperm reactivation after UV-irradiation in sturgeon and propose a way to eliminate the negative influence on the induction of gynogenesis.

2. Material and methods

This experiment was conducted at the Genetic Fisheries Center, Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Spermiation was induced in males by injecting carp pituitary extract (4 mg/kg) 24-h before sperm collection, according to Linhart et al. (2000). Motility of the collected sperm was investigated. Sperm from two males with a motility of > 70% was selected for further study. Ovulation in females was induced by hormonal stimulation with 0.5 mg/kg of a carp pituitary suspension 42-h before expected ovulation and 12-h later with a resolving dose of 4.5 mg/kg (Gela et al., 2008). Ovulated eggs were sampled by microsurgical incision of oviducts, as described by Štěch et al. (1999).

The UV irradiation for sperm was provided by a UV crosslinker CL-1000 (254 nm, Ultra-Violet Products Limited, England), with a light intensity of 45 W/m^2. Sperm was diluted five times in artificial medium with ionic concentrations similar to native seminal fluid, according to Psenicka et al. (2008). Aliquots of 1000 µl of diluted sperm were irradiated with UV-C light on Petri dishes (diameter, 90 mm) at doses of 50, 100, 150, 200, and 250 J/m^2 for gynogenesis activation and at doses of 500 and 1000 J/m^2 for the comet assay. The Petri dishes were kept on ice gently agitated at 50 rpm during irradiation. After irradiation, the sperm was collected from the Petri dishes, placed in covered Eppendorf tubes, and kept on ice. To investigate influence of photoreactivation, 1 ml of diluted sperm was poured on the Petri dish placed on ice under an incandescent bulb (1350 Lm) at a distance of 50 cm or under infrared bulb (Exo Terra Infrared Heat Glo 100 W) at the same distance. The Petri dishes from the control group were covered with aluminum film. The sperm suspension after the treatment was diluted with prechilled PBS (1 : 250) and stored at 4 °C.

For fertilization, 20 ml of water (15 °C) was added to each sample (200 ml of sperm), and this suspension was immediately added to the eggs (4 g), according to Gela et al. (2008). The eggs were fertilized for 2 min with gentle stirring at 16 °C, and then distributed into three Petri dishes. After sticking of the eggs, the Petri dishes were immersed in incubators and maintained at 16 °C until hatching. All hatched larvae were assessed for ploidy level by measurement of the relative DNA content by a Partec flow cytometer CCA (Partec GmbH, Germany), according to Lecommandeur et al. (1994). Untreated larvae of the paleotetraploid (4n) sterlet *Acipenser ruthenus* were used as a control group.

For single-cell electrophoresis, agarose gel 0.8% was melted at 90 °C and chilled to 37–40 °C. Agarose was mixed with diluted sperm at ratio of 9 : 1. Immediately after mixing, 50 µl

of suspension was pipetted to each well on a Teflon printed diagnostic slide and spread over the well. Slides were stored at 4 °C until the agarose had solidified and then immersed in the lysis solution prepared according to the OxiSelect Comet Assay protocol with the addition of Proteinase K (1 mg/ml of lysis solution). Slides were incubated in the lysis solution for 10 h at room temperature. After incubation, the lysis solution was carefully aspirated and replaced with prechilled TBE electrophoresis solution (90-mM Tris Base, 90-mM Boric acid, 2.5-mM EDTA; pH 8.0). After 5 min, the TBE buffer was replaced with new TBE buffer and then slides were carefully transferred to the electrophoresis chamber with the cold electrophoresis solution. Electrophoresis was performed for 20 min at 1 V/cm, and then the slides were transferred to a small container with prechilled distilled water for 5 min. Distilled water was carefully aspirated and slides were air-dried. Once the agarose slide was completely dry, 20 µl/well of Vista Green DNA Staining Solution (OxiSelectST; Cell Biloabs, Inc. USA) was added. Slides were observed under Olympus Fluoview microscope using filters with 450–480-nm excitation wavelengths and recorded on the SONY DXC-9100D videocamera. The DNA damage in spermatozoa was evaluated using CASP 1.2.3 freeware (Comet Assay Software Project lab, Poland). To induce partial gynogenesis, eggs were activated with sperm subjected to UV-C light followed by visible light treatment.

All values were expressed as mean ± S.D. Data were tested for normal distribution and analyzed by the Statistica 9 software, by one-way ANOVA, followed by Tukey's test for comparisons of means ($p < 0.05$).

3. Results and Discussion

DNA reactivation in UV-irradiated sperm as measured by the comet assay resulted in a significant level of DNA restoration after irradiation by an incandescent bulb for 10 min (Fig. 1).

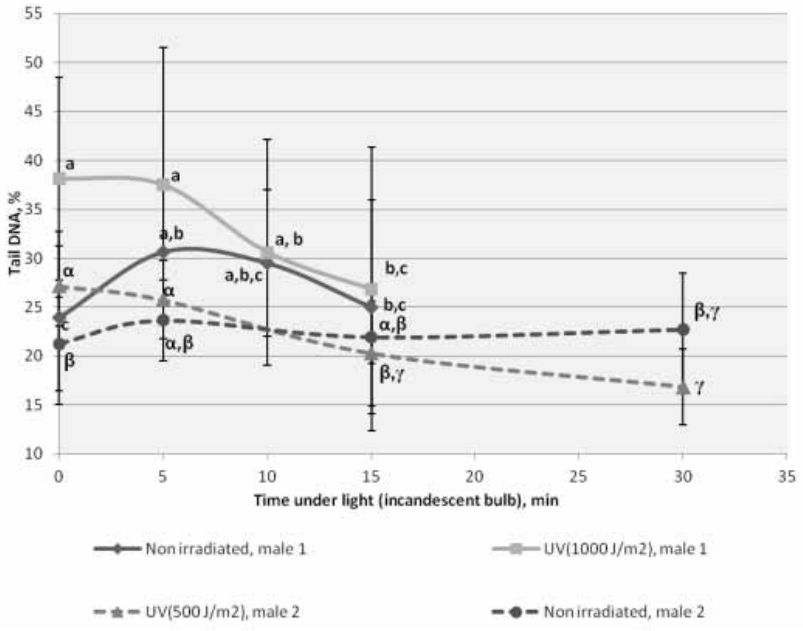

Fig. 1. *Level of DNA damage relative to the duration of incandescent bulb exposure in sperm irradiated with UV-C light at doses of 0, 500, and 1000 J/m².*
a, b, c, d; α, β, γ. $p < 0.05$ (by ANOVA and Tukey's test)

The level of DNA restoration was higher in sperm irradiated with a higher dose (1000 J/m^2); hence, the influence of reactivation was particularly significant when high doses of UV were applied. Nonirradiated sperm showed moderately increased DNA damage level after short-term exposure to visible light; however, longer exposure induced a return to the native level of DNA damage. The red light did not cause DNA repair and instead a moderate increase of DNA damage level was observed (Fig. 2). This damage might be probably caused by heating the sperm, and thus, the light source should be placed at a sufficient distance from the sample. On the basis of these results, it is suggested that red light should be used to illuminate work stations while handling sperm samples for gynogenesis induction.

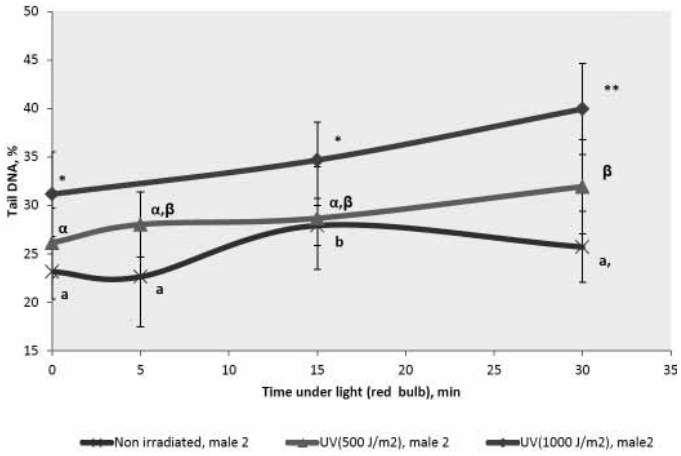

Fig. 2. *Level of DNA damage relative to the duration of red light (600–750 nm) exposure to sperm irradiated with UV-C light at doses of 0, 500, and 1000 J/m^2.*
*a, b; α, β; *,**p. < 0.05 (by ANOVA and Tukey's test)*

Induction of gynogenesis with UV-C irradiation followed by exposure to visible light resulted in a significantly different hatching rate after 10 min of exposure to an incandescent bulb (1350 Lm, 50 cm) and caused derivations from the typical Hertwig effect (Fig. 3).

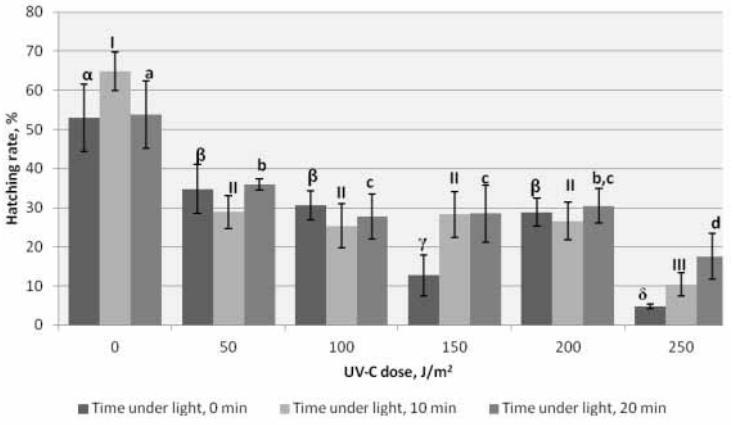

Fig. 3. *Hatching rate relative to the UV-C dose used to induce gynogenesis in sperm exposed to an incandescent bulb (1350 Lm, 50 cm) for 0, 10, and 20 min.*
** a, b, c, d; α, β, γ, δ; I, II, III. p < 0.05 (by ANOVA and Tukey's test)*

We assumed that these deviations were caused by light-dependent DNA restoration. This assumption was indirectly confirmed by a larval ploidy investigation (Fig. 4).

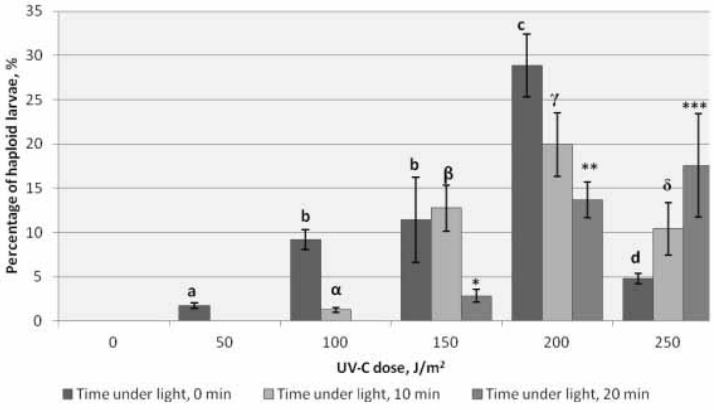

Fig. 4. *Hatching rate of haploid larvae (putative gynogenotes) relative to the UV-C dose used to induce gynogenesis in sperm exposed to an incandescent bulb (1350 Lm, 50 cm) for 0, 10, and 20 min.*
** a, b, c, d; α, β, γ, δ; *, **, ***p. < 0.05 (by ANOVA and Tukey's test)*

The maximum percentage of putative gynogenotes in progeny was shifted after the exposure of sperm to the incandescent bulb. These results indicate that the exposure of UV-C irradiated sperm to visible light can significantly affect induced gynogenesis.

4. Conclusions

DNA reactivation in UV-irradiated sperm measured by the comet assay showed significant levels of DNA restoration in sperm maintained under an artificial light condition for >10 min (incandescent bulb, 1350 Lm, 50 cm). The level of DNA restoration was higher in sperm irradiated with higher doses; hence, the influence of reactivation was particularly significant when high doses of UV were applied. In addition, the results of the effects of light source with wavelength longer than 600 nm on UV-irradiated sperm indicated that this light did not induce light-dependent DNA reactivation. Therefore, the use of red light to illuminate work stations while performing gynogenesis induction is suggested.

Acknowledgments

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CHAPTER 5

USE OF FLOW CYTOMETRY TO ASSESS THE SUCCESS RATE OF INTERSPECIFIC GYNOGENESIS INDUCTION AND TO SEPARATE NONGYNOGENETIC PROGENY OF STURGEONS

Lebeda, I., Steinbach, C., Bytyutskyy, D., Flajšhans, M., 2014. Use of flow cytometry to assess the success rate of interspecific gynogenesis induction and to separate nongynogenetic progeny of sturgeons. (manuscript)

USE OF FLOW CYTOMETRY TO THE ASSESS SUCCESS RATE OF GYNOGENESIS INDUCTION AND TO SEPARATE NONGYNOGENETIC PROGENY OF STURGEONS

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Abstract

In the present study, we demonstrated that ploidy analysis can be an effective method for separating sturgeon gynogenetic progeny induced by heterologous sperm with different ploidy levels. Gynogenesis was induced in tetraploid Siberian sturgeons, *Acipenser baerii*, with UV-C-irradiated sperm from the diploid sterlet *Acipenser ruthenus*. The success of the gynogenesis induction and diploidy restoration treatment were estimated based on the assessment of the progeny ploidy level. The survival rates of gynogenetic progeny were comparable with those of the hybrids, i.e., 19.2% and 8.8% of fertilized eggs at 6 months after hatching in the hybrid and gynogenetic groups, respectively. The high survival rates and effectiveness of ploidy restoration were demonstrated after heat shock treatment with 34 °C for 2 min at $0.25\tau_o$ after egg activation.

Keywords: *functional ploidy; gynogenesis; sturgeon; flow cytometry*

Introduction

Gynogenotes of some sturgeon and paddlefish species may help to primarily obtain female progeny (Van Eenennaam, 1997; Keyvanshokooh & Gharaei, 2010; Shelton & Mims, 2012). The methods used for the artificial induction of gynogenesis include three main procedures: i) inactivation of DNA in spermatozoa, ii) activation of eggs, and iii) restoration of zygote diploidy. DNA inactivation can be achieved using different chemical and physical methods (Ihssen et al., 1990; Komen & Thorgaard, 2007), but the most common method is to irradiate sperm with shortwave UV-C (254 nm) light in order to damage highly sensitive DNA. However, complications in the optimization of UV treatments, such as the low UV transparency of sperm, variability in the spermatozoa concentration between males, and the sensitivity of the sperm motility apparatus to UV light lead to high variability in the proposed optimal parameters (Mims & Shelton, 1995). Thus, the reported doses of UV-C irradiation used to inactivate DNA in sturgeons vary from 135 J/m^2 (Recoubratsky, 2003) to 2838 J/m^2 (Saber et al., 2008).

There are several methods for optimizing UV irradiation of sperm. Dietrich et al. (2005) demonstrated that comet assay can be used to monitor the effectiveness of fish sperm DNA inactivation by UV irradiation, particularly when used in combination with spermatozoa motility analyses. Unfortunately, this method is useful for comparing different methods of sperm inactivation, but it cannot predict the threshold required for sperm DNA inactivation. The most common method is to induce gynogenesis using sperm irradiated with different doses of UV. In this case, the percentage of gynogenotes among the progeny can be estimated with different methods. The most reliable method for evaluating the efficiency of gynogenesis induction is a microsatellite DNA assay. However, this method is expensive, laborious, and

requires samples of the parental tissues. Another possibility is to eliminate the third step of gynogenesis induction, i.e., zygote diploidy restoration. In this case, all the haploid larvae can be determined as putative gynogenotes. This method has many advantages such as it is relatively inexpensive, easy to perform, and does not require samples of the parental tissues, and it also eliminates the influence of another variable, i.e., the ploidy restoration treatment. The easiest method for separation is interspecific gynogenesis. The use of heterologous sperm for gynogenesis induction has been reported for many fish species i.e., Wels catfish *Silurus glanis* (Fopp-Bayat, 2010), grass carp *Ctenopharyngodon idellus* (Zhang et al., 2011), paddlefish *Polyodon spathula* (Mims et al., 1997), and shovelnose sturgeon *Scahirhynchus platorynchus* (Mims & Shelton, 1998). Unfortunately, because of high phenotype variability of hybrid progeny separation of gynogenotes according phenotype markers cannot be a reliable method. Nevertheless, the progeny can be separated if the hybrids are nonviable or based on the ploidy level if the ova have been activated by heterologous sperm with a different ploidy level.

The ploidy level of sturgeons is different than that in teleost and the answer remains unclear to the question of sturgeon ploidy level system. Investigation of the sturgeon genome structure and size by karyotyping or flow cytometry classified extant species into three groups: those possessing approximately 120, 250, and 360 (Vasil'ev, 1987; Birstein et al., 1993). These species are called evolutionary tetraploid, octaploid, and dodecaploid, respectively (Flajshans & Vajcova 2000); however, according another study these species are functional diploid, tetraploid, and hexaploid. In addition, the ploidy system of sturgeon species is complicated because of the high plasticity of the ploidy level that originates from possible hybridization between sturgeon species with different ploidy and spontaneous polyploidyization in progeny such as the occurrence of triploids fish in populations because of occasional failure of the extrusion of the second polar body of the fertilized egg (Flajshans, 2006).

The aim of the present study was to assess the suitability of ploidy analyses for determining and separating gynogenotes, and to investigate the possibility of using heterologous sperm for inducing gynogenesis in the widely used sturgeon model species *Acipenser baerii* with UV-C-irradiated sperm of sterlet.

Material and Methods

The experiments were conducted at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Spermiation was induced in males, by a previous method (Linhart et al., 2000). Ovulation was induced in females by hormonal stimulation with carp pituitary suspension, and the ovulated eggs were collected by microsurgical incisions in the oviducts, as described previously (Lebeda et al., 2013).

The UV-C irradiation of sperm was achieved using a UV crosslinker CL-1000 (254 nm, Ultra-Violet Products Limited, England) with a light intensity of 45 W/m^2. Sperm dilutions were prepared using artificial medium of pH 8.1, a $Ca2^+$ concentration of 0.16 mM, Na^+ 20.1 mM, K^+ 1.5 mM, and osmolality adjusted using the TRIS base to 80 mosmol/kg, according to Psenicka et al. (2008). Aliquots of diluted sperm (1:4, 500 μl) were irradiated with UV-C light in Petri dishes (diameter = 90 mm) at a dose of 200 J/m^2, according to Lebeda et al. (2014). The Petri dishes were agitated gently at 50 rpm throughout the irradiation process.

After irradiation, the sperm were maintained in the dark at 4 °C until further use. The motility of irradiated sperm was evaluated. For egg activation, 400 ml of water (16 °C) was added to 3 ml of sperm (six aliquots) and this suspension was added immediately to 100 g of eggs, which corresponded to approximately 5000 eggs. The duration of activation was 2 min and the suspension was gently stirred at 16 °C during the process. After fertilization, the eggs

were washed with water, immersed in desticking solution (fine clay suspension 20 g/l), and mixed gently by hand (Gela et al., 2003). The eggs were maintained in the desticking solution at 16 °C until the ploidy restoration treatment. Three experimental groups were produced, i.e., control hybrids, triploid hybrids, and the gynogenetic group. The first group was a result of fertilization of Siberian sturgeon eggs with untreated sterlet sperm and cultivation at constant temperature. The Triploid hybrids were produced by fertilization of eggs with untreated sperm and application of temperature shock. Gynogenesis was induced by fertilization of eggs with UV-irradiated sperm and application of ploidy restoration treatment.

The temperature shock treatment was applied at $0.25\tau_o$, which corresponded to 18 min of egg incubation at 16 °C (Dettlaff et al., 1993), according to Mims & Shelton (1998). The eggs were immersed in the desticking solution for 2 min at 34 °C, according to previously conducted optimization (unpublished data). Next, the heat-shocked eggs were washed with 4 l of cold water (16 °C) and immersed in the desticking solution at 16 °C for 45 min. The samples were separated into two incubation jars, each of which contained approximately 50 g of eggs and were incubated at 16 °C.

The hatched larvae were counted on day 10 after hatching commenced. At this stage, 20, 30, and 50 larvae were used for ploidy measurements from the control hybrid, triploid hybrid, and gynogenetic groups, respectively. Subsequent samples were collected after 30 and 180 days. The survival rate was calculated relative to the amount of fertilized eggs. The juvenile mortality rate was calculated as the percentage of fish that died during growth relative to the amount of fish at the beginning of the feeding stage. The ploidy of larvae was estimated using the caudal fin. Tissue samples were minced and incubated in Nuclei Extraction Buffer (CyStain DNA 2-step, PARTEC). The ploidy level of 6-month-old (180 days) juveniles was estimated using blood samples preserved in saline solution (2 drops/ml). Nuclei in separated and permeabilized cells were stained with fluorescent DNA dye, DAPI (4',6-diamidino-2-phenylindol). Partec Cube 8 flow cytometer was used to estimate relative DNA content per cell. The untreated tetraploid *Acipenser ruthenus* larvae were used as reference.

Results and discussion

The induction of gynogenesis in Siberian sturgeons using UV-irradiated sterlet sperm had a relatively high hatching rate of 39.0% on day 10 after hatching (Fig. 1).

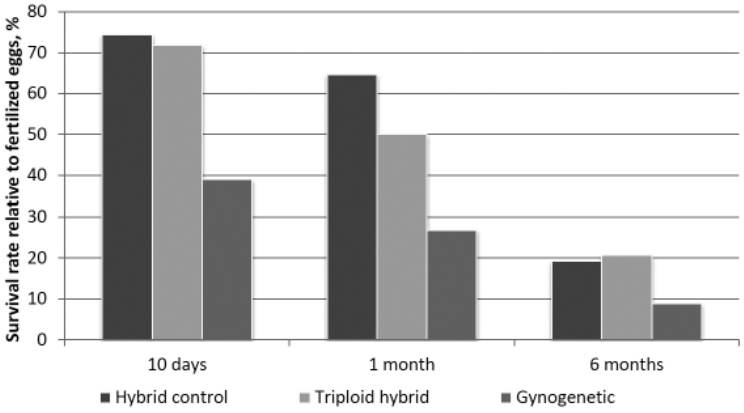

Fig. 1. *Progeny survival rates relatively to the number of eggs (5000 eggs) at 10, 30, and 180 days after hatching.*

A low mortality rate was also observed during the early development of gynogenetic juveniles, where up to 8.8% of the fertilized eggs developed into gynogenetic fish at 6 months, which corresponded to a juvenile mortality rate of 77.4%, whereas the mortality rate in the control hybrid group was 74.2%. In the triploid hybrids group at 6 months, the survival rate was even higher than that in the control hybrid group. Therefore, we considered that the diploidy restoration treatment during the second meiotic division did not have any substantial effect on the survival rate. We used a relatively low dose of UV-C light (200 J/m^2) to inactivate the DNA in spermatozoa compared with that used by Saber et al. (2008; *Acipenser stellatus*, 2838 J/m^2), Van Eenennaam et al. (1996; *Acipenser transmontanus,* 2160 J/m^2), and Flynn et al. (2006; *Acipenser brevirostrum*, 1200 J/m^2). Despite using a low UV-C dose, the number of nongynogenetic fish was low in the gynogenetic group (Table 1), i.e., up to 4% on day 10 after hatching, 3.3% on day 30, and 2.5% on day 160, and we can thus conclude that these doses are sufficient for the induction of gynogenesis. The low proportion of nongynogenetic juveniles may be explained by the effect of UV-C light on sperm DNA during its inactivation, which could have caused mutations in the nongynogenetic progeny and increased their mortality.

Table 1. *Ploidy levels of the progeny on days 10, 30, and 180 after hatching. the control hybrid group: ♀ Sib. Sturgeon (4n) × ♂ sterlet (2n); the triploid hybrid group: ♀ Sib. Sturgeon (4n) × ♂ sterlet (2n) + heat shock; the gynogenetic group: ♀ Sib. Sturgeon (4n) × ♂ sterlet (UV-irradiated; 2n) + heat shock.*

Group	Days after hatching	Sample number	Ploidy 3n (ca.180 chromo-soms)	Ploidy 4n (ca.240 chromo-soms)	Ploidy 5n (ca.300 chromo-soms)	Aneuploid
Control hybrid	10	20	20	–	–	–
Triploid hybrid	10	30	2	–	27	1
Gynogenotes	10	50	2	47	–	1
Control hybrid	30	10	10	–	–	–
Triploid hybrid	30	20	2	–	18	–
Gynogenotes	30	30	1	29	–	–
Control hybrid	180	30	30	–	–	–
Triploid hybrid	180	30	1	–	29	–
Gynogenotes	180	80	2	76	–	2

The success of paternal genome inactivation and ploidy restoration were assessed by sampling 20–50 fish from each group (Fig. 2). The relative DNA content showed clear separation of the gynogenetic group on three subgroups with ploidy levels three, four, and five.

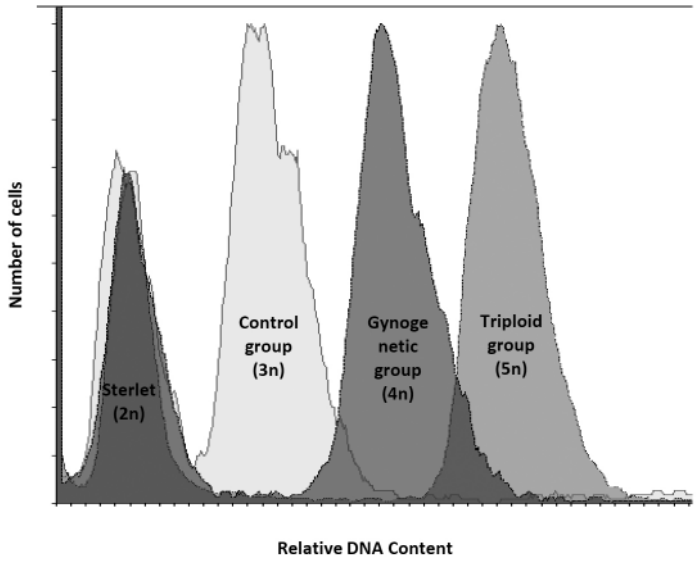

Fig. 2. *Ploidy results comparison. Native sterlet (2n) tissue was added to each sample as a reference. Control group: ♀ Sib. Sturg. (4n) × ♂ sterlet (2n); Triploid hybrid group: ♀ Sib. Sturg. (4n) × ♂ sterlet (2n) + heat shock; Gynogenetic group: ♀ Sib. Sturg. (4n) × ♂sterlet (UV-irradiated; 2n) + heat shock. The data were processed on Flowing Software 2.5.1.*

Usually, microsatellite studies on gynogenesis induction success are limited because of the amount of samples and the expensive and time-consuming procedures. Flow cytometry analysis of ploidy is much cheaper and easier to perform than microsatellite assays, and they do not require samples from parental tissues. In addition, this method allows the evaluation of survival rates of gynogenetic and non gynogenetic parts of progeny without physical separation of progeny, and further, the separation of fish by PIT tagging and ploidy analysis of blood..

Conclusion

The induction of gynogenesis using heterologous sperm is a promising method for gynogenetic sturgeon production. The induced gynogenesis in Siberian sturgeon using heterologous sperm yielded an adequate survival rate of up to 39% on day 10 after hatching. The application of low doses of UV irradiation (200 J/m^2) to sperm was effective in inactivating DNA and only 4% nongynogenetic fish were found on day 10 after hatching. As a result, up to 8.8% of the fertilized eggs developed into gynogenetic juveniles at 6 months, which can be effectively isolated by PIT tagging and ploidy analysis. In addition, the results of the study suggest the high rate of successful diploidy restoration and a small contribution of this treatment in a final mortality rate.

Acknowledgments

This study was supported in part by projects CENAKVA CZ.1.05/2.1.00/01.0024, LO1205, and GAJU 114/2013/Z and by the GAJU 086/2013/Z project. The results of the project LO1205 were obtained with a financial support from the MEYS of the CR under the NPU I program.

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CHAPTER 6

OPTIMIZATION OF THE TETRAPLOIDIZATION PROTOCOL IN STERLET, *ACIPENSER RUTHENUS*

Unpublished data

Optimization of the tetraploidization protocol in sterlet, *Acipenser ruthenus*

Introduction

The induction of tetraploidy is based on suppression of the first cleavage division that leads to the duplication of the zygote chromosome number (Pandian & Koteeswaran, 1998). An example of an application of this treatment is the production of tetraploid *Oncorhynchus mykiss* for the further production of triploids (Thorgaard, 1981). This treatment is similar to the mitotic ploidy restoration treatment used for the production of highly homozygous gynogenetic individuals called doubled haploids, e.g., *Cyprinus carpio* (Wu et al., 1986), *Plecoglossus altivelis* (Taniguchi et al., 1990), *Oreochromis niloticus* (Peruzzi et al., 1993), or the restoration of the ploidy level during androgenesis, as done for *Oncorhynchus mykiss* by Scheerer (1986). Mitotic tetraploidization usually has a low success rate and leads to the appearance of malformations of larvae development. In sturgeons, a similar treatment was used for the restoration of diploidy during androgenesis induction, such as during dispermic androgenesis induction in Siberian sturgeon, *Acipenser baerii Brandt* (Grunina, 1991) and in starry sturgeon, *Acipenser stellatus* (Recoubratsky, 1996). This treatment focused on the fusion of sperm nuclei after polyspermic fertilization (Grunina et al., 2011) and is applied at 1.4–1.6 τ_o after fertilization (Grunina, 1991 and 2011; Recoubratsky, 1996).

In this study, we optimized the protocol of temperature shock for mitotic tetraploidization. For the treatment, we applied temperatures commonly used in triploidization treatment and in the fusion of sperm nuclei during dispermic androgenesis in sturgeons, and time range from the moment of extrusion of the second polar body (0.6 at 16 °C) to the prophase of the first mitotic division (1.6 τ_o at 16 °C; Dettlaff et al., 1996).

Materials and methods

The fish were obtained from the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Spermiation was induced in males by previously published method (Linhart et al., 2000). Ovulation was induced in females by hormonal stimulation with carp pituitary suspension and the ovulated eggs were collected by microsurgical incisions in the oviducts, as described previously (Štěch et al., 1999; Gela et al., 2008). For fertilization, 20 ml of water (15 °C) was added to 125 µl of sperm, and this suspension was immediately added to 4 g of eggs, according to Gela et al. (2008). The eggs were fertilized for 2 min with gentle stirring at 16 °C, and then distributed into three Petri dishes. After sticking of the eggs, the Petri dishes were immersed in incubators and maintained at 16 °C until the heat shock treatment. The heat shock treatment parameters are described in Table 1. After the heat shock treatment, the Petri dishes were immediately placed in an incubation system at 16 °C and incubated until hatching. To prevent fungal infection, dead eggs were removed after 2-days of incubation when the neural tube could be clearly observed. The ploidy level of up to 20 hatched larvae from each group was analyzed using the caudal fin. Tissue samples from the fin were minced and incubated in Nuclei Extraction Buffer (CyStain DNA 2-step, PARTEC). Nuclei in separated and permeabilized cells were stained with fluorescent DNA dye, DAPI (4',6-diamidino-2-phenylindol). Partec Cube 8 flow cytometer was used to estimate the relative DNA content per cell. The untreated diploid *Acipenser ruthenus* larvae were used as reference.

Table 1. *Tested parameters of the heat shock treatment. The duration of one mitotic cleavage division τ_o is approximately 63 min for sterlet at 16°C, according to Dettlaf et al. (1993).*

Temperature, °C	Duration, min	Starting time, min	Starting time, τ_o
34	2	56	0.88
		74	1.75
		87	1.38
		99	1.57
	3	56	0.88
		74	1.75
		87	1.38
		99	1.57
37	2	50	0.79
		56	0.88
		74	1.75
		87	1.38
		99	1.57
	4	50	0.79
		56	0.88
		74	1.75
		87	1.38
		99	1.57

Results and Discussion

Application of the mitotic heat shock treatment at an intensity similar to that commonly used in the triploidization treatment (temperature 34 °C for 2 min), resulted in a relatively high hatching rate (approximately 70%); however, the tetraploidization efficiency was extremely low, and thus only one tetraploid larva was found (in the group treated for 74 min after activation for 2 min). Several larvae showed ploidy level 6n, which might be a result of retarded early development and meiotic ploidy restoration. Increasing the duration of the shock treatment to 3 min had no substantial effect on the hatching rate, although it did not increase the efficiency of the treatment, and thus, no tetraploid larvae were found.

In contrast, treatment with higher temperature (37 °C) was sufficient to primarily produce tetraploid progeny; however eggs hatched only in the groups treated for 2 min and the hatching rates were substantially lower than those after the application of the heat shock treatment at 34 °C (Fig. 1).

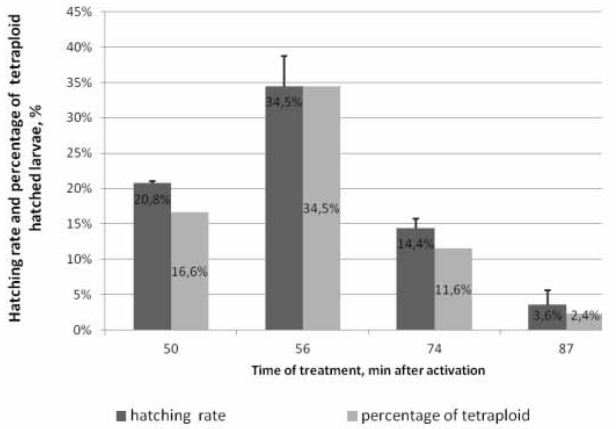

Fig. 1. *Hatching rate and the percentage of tetraploid hatched larvae relatively to the time of the heat shock treatment. Eggs were immersed in a water bath at 37 °C for 2 min. (τ_d approximately 63 min at a cultivation temperature 16 °C, according to Dettlaf et al., 1993). Groups treated with heat shock for 4 min did not hatch.*

The highest efficiency of tetraploidization was observed after the heat shock treatment at 37 °C which was initiated 56-min after activation for 2 min. This treatment resulted in 34.5 ± 4.3% of hatched eggs as well as all processed larvae were tetraploid (n = 25). This survival rate was relatively high taking into account that tetraploidy was obtained only in few fishes and mostly with a low efficiency (Pandian & Koteeswaran, 1998). Probably, tetraploidization can be induced more easily in sturgeons because of the plasticity of their ploidy system.

This experiment represents a great interest because of the possible application in the production of triploids, induction of gynogenesis and androgenesis with diploid gametes, and for the restoration of ploidy during gynogenesis, leading to the production of highly homozygous gynogenetic progeny. On the other hand, the efficiency of tetraploid larvae production is questionable because of the occurrence of malformations in the overwhelming majority of tetraploid larvae (Fig. 2), which could originate from the influence of heat shock – a known source of malformations. Malformations in tetraploid larvae can lead to further mortality; hence survivability of tetraploids should be additionally studied.

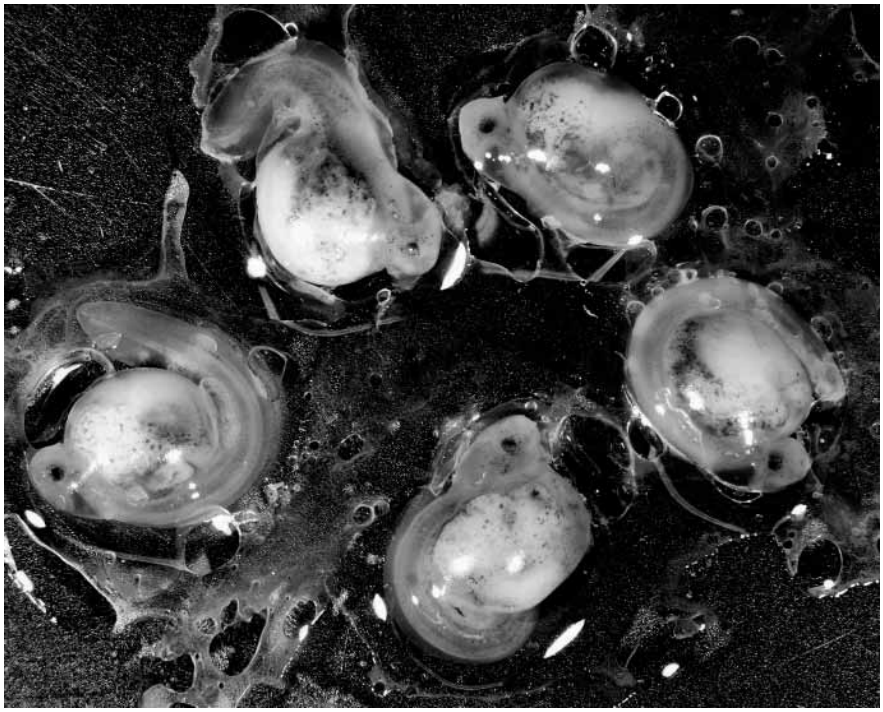

Fig. 2. *Typical malformation in tetraploid larvae, 1 day after hatching.*

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CHAPTER 7

GENERAL DISCUSSION

Gynogenesis in sturgeons

As a potentially powerful method for sex determination system research and monosexual shoal production tool, gynogenesis has been under detailed investigation. After decades of studies, low survival rates and high variability of results were the main problems of gynogenesis implementation in aquaculture, e.g., the optimal UV dose for the inactivation of DNA in sturgeon sperm proposed by different authors varies from 135 J/m^2 (Recoubratsky et al., 2003) to 2838 J/m^2 (Saber et al., 2008). It became obvious that the development of the gynogenesis induction protocol is not a trivial task and requires different approaches to each species and specimen. Therefore, the necessity for a preliminary study of the reaction of sperm to DNA inactivating agents was required (Lebeda et al., 2014a). The study conducted on the influence of UV light, as a conventional DNA inactivating agent, on spermatozoa motility showed strong dependence of irradiation heterogeneity of the sample on its dilution ratio, explained by the investigation of the UV-light absorption level in sperm and confirmed by the induction of partial gynogenesis. The importance of dilution was emphasized by other authors, e.g., by Mims and Shelton (1995) who, using shovelnose sturgeons (*Scaphirhynchus platorynchus*), described the dependence of spermatozoa lethal UV dose on sperm transmittance and used it for calculating UV treatment. Homogeneity can be achieved by preliminary spectrophotometry investigation of sample transparence to UV light or by high dilution of the sample. The former is time consuming and requires a spectrophotometer at spawning facility. The latter involves dramatically increasing the volume of irradiated samples, which makes the irradiation process laborious and might cause problems with further activation of sperm, thus increasing the variability of the experiment. In our study, we used dilution ratios of 1 : 4 to 1 : 6, depending on sperm density, and used sample layer depth ranging from 0.2 to 0.5 mm. This dilution is a compromise between the need of UV-irradiation homogeneity and the increase of sample volume. On the other hand, according to the investigation of sperm UV transparency, the sensitivity of the motility apparatus and partial gynogenesis induction, these UV-irradiation parameters were sufficient for homogeneity of irradiation.

The inactivation of the paternal genome by chemical agents was proposed as an alternative for the large-scale induction of gynogenesis. In our previous study, (Lebeda et al., 2014b) we investigated several candidates for chemical genome inactivation. The direct damaging agent showed low selectivity of influence, and probably because of high chemical activity they interacted with the other system of spermatozoa, particularly the motility apparatus. On the other hand, damaging agents that act by intercalation of chemicals in the DNA structure showed higher efficiency. Unfortunately, this approach required irradiation the sample with long-wave UV-A light that only decreases spermatozoa motility (Lebeda et al., 2014b). Nevertheless, by this method we managed to obtain up to 20% of gynogenetic larvae in relation to the amount of fertilized eggs, which was sufficient taking into account the high fertility of sturgeons. This method can be applied to larger volumes of sperm because of the higher transparency of sperm in the UV-A region.

Another possible source of gynogenesis result variability is the photoreactivation of DNA in spermatozoa. Photoreactivation during gynogenesis induction in sturgeons was reported for the first time by Recoubratsky in 2003, who reported a shift of the Hertwig effect in sperm subjected to daylight compared with those kept in the dark after UV irradiation. His study qualitatively indicated the presence of this effect, but results were obscured because of the variability of gynogenesis induction, as described above. Therefore, in our study on photoreactivation, we used the comet assay to estimate quantitatively the level of DNA

photoreactivation in sperm. Substantial difference in DNA damage levels after subjecting UV-irradiated sperm to incandescent bulb, particularly after high doses of UV, indicated importance of protecting samples from visible light. In contrast, red light with wavelength of more than 600 nm did not induce DNA reactivation. Probably, the red light had insufficient energy to activate enzymes responsible for light-dependent DNA repair. As a result, the use of red bulbs for illuminating working stations was suggested for the convenient handling of UV-irradiated sperm.

The successful application of the meiotic heat shock treatment for ploidy restoration, similar to triploidization treatment, was described by many authors. With this treatment we managed to obtain a high survival rate, triploid sterlet hatching rate of up to 90–80%, compared with those of the control. However, hatching rate appearance of malformations in progeny after hatching rate application of heat shock for triploidization was reported in different species (Piferrer et al., 2009). From this point of view, the application of pressure shock is more promising. On the other hand, little is known about pressure shock in sturgeons; in addition, this procedure requires desticking of eggs, expensive equipment, and skills. Therefore, we believe that pressure shock methods are worth studying and could be used in large-scale production but might seem inconvenient compared with heat shock, especially in the case of sticky eggs and small-scale experiments. Mitotic rediploidization has not been applied in gynogenesis induction because of its low efficiency and its substantially low survival rate of the treated embryos. Nevertheless, we optimized this treatment in sterlet to establish methods of tetraploid sturgeon production; as a result, we succeeded in producing up to 34% of tetraploid larvae. Unfortunately, most of the larvae exhibited body malformations, probably of the same nature as those described when using heat shock for triploidization.

Future of gynogenesis

Demands for reliable methods for improving sturgeon aquaculture warrant studies on gynogenesis; however, the application of gynogenesis appears to be a prospect of the distant future. The positive aspects of gynogenesis application reduced because of the ZW/ZZ sex determination system in sturgeons, which resulted in presence of both female and male gynogenetic progeny. In addition, despite the constant improvement of our understanding of gynogenesis in sturgeons, there is still a lack of knowledge with regard to the effect of gynogenesis induction on the ontogenesis of fish; therefore, quick introduction of gynogenesis in sturgeon aquaculture is questionable. Clearly, we expect the presence of malformations and decrease in fitness because of the rise of homozygosity, i.e., the increase of the genetic load. In our study, we found a low percentage of body malformations in gynogenetic progeny compared with those in triploids; therefore, we believe that the observed malformations are related to temperature shock in the same way as it was repeatedly described after triploidization in other fish species and summarized in the review of Piferrer et al. (2009). Another obstacle to the introduction of gynogenesis in sturgeon aquaculture is the low and variable survival rate of gynogenetic progeny. A low survival rate can be partially overcome by extensification of larvae production that is usually is not problematic because of the high fertility of sturgeons. Commonly used UV-C treatment of sperm is inconvenient for large-scale gynogenotes production because of the high absorption of UV in sperm. Therefore, we focused on methods of large-scale gynogenesis induction, such as chemical inactivation of parental DNA (Lebeda et al., 2014b), as described in Chapter 3. Despite the described the low efficiency of this method, the possibility of applying it to large volumes of sperm, up to few liters, can make it a method of choice for future application in aquaculture. The other key point is the presence of nongynogenetic fish in progeny. As a result, it leads to difficulties in

protocol optimization processes and necessitates the use of microsatellite analysis in order to verify the true absence of paternal genetic information in the genome of progeny. The induction of gynogenesis using heterologous sperm of another species, as described by Mims et al. (1995) and Chapter 5, opens a wide field for study, e.g., the induction of interspecific gynogenesis in species with nonviable hybrids can substitute expensive, time consuming, and laborious microsatellite assays. In addition, interspecific gynogenesis using gametes of species differing in ploidy levels can be used for gynogenetic progeny separation, according to the method described in Chapter 5.

According to the hypothesis of female heterogamety in sturgeons, the appearance of WW superfemales in gynogenetic progeny can be expected. Only females should be present in the progeny of these super females; hence, the presence of these fish can greatly improve the efficiency of sturgeon aquaculture. Unfortunately, because of the absence of known genetic markers of sex and unknown sex chromosomes, these individuals can be separated only by investigating the sex in their progeny, and hence such a study would last for two generations approximately from 7 to 20 years depending on each sturgeon species.

The underlying mechanisms of gynogenesis in sturgeons, particularly paternal genetic information blocking during the activation of eggs, have not been thoroughly investigated. Probably, it is similar to those during gynogenesis in the sea urchin (*Lytechinus pictus*) or crucian carp (*Carassius auratus*). In these species, authors described the failure of nuclear envelope breakdown as a result of UV irradiation of sperm or insemination with interspecific sperms (Yamashita et al., 1900; Sluder et al., 1995). Therefore, it is believed that DNA damage of spermatozoa causes a block at the checkpoint of the nuclear envelope breakdown process, and hence, the paternal nuclear envelope has substantially lags the breakdown, leading to the development of the embryo solely from the maternal pronucleus. Unfortunately, because of the size and color of the sturgeon egg, it is hard to visually observe pronucleus breakdown, as observed in the aforementioned species with transparent eggs.

Sturgeons – the critically endangered fish group – are one of the most striking examples of the impact of overexploitation among fish. In last few decades, focus was shifted to aquaculture production of sturgeon products, which was one of the key factors for increased scientific interest in sturgeon studies. Taking into account that the interest in aquaculture was almost solely directed for caviar production, chromosomal manipulation became a key interest as a way to the increase ratio of females in progeny. Despite decades of experiments, with the induction of gynogenesis in sturgeons, issues such as low survival rates, high variability of results, and the lack of knowledge about genetic and fitness consequences of this treatment hinder its adaptation for large-scale production. Recent studies showed that the optimization of gynogenesis induction protocols is a challenging, species-specific, multifactorial task, requiring the control of each step of gynogenesis induction, and accounting for factors like photoreactivation. Nevertheless, methods and protocols described in the present study allowed gynogenesis induction in sturgeons with survival rate sufficient for aquaculture. In addition, methods of chemical inactivation of DNA in sperms suited for large-scale production of gynogenotes were proposed. Part of the study focused on protocols that are more universal and eliminate the variability of results, e.g., by homogenous UV irradiation and prevention of DNA photoreactivation.

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ENGLISH SUMMARY

Optimization of chromosomal manipulations in Acipenserids

Ievgen Lebeda

Highly profitable black caviar market and the depletion of wild sturgeon stocks warrant improvements in sturgeon aquaculture. Therefore, chromosomal manipulations, particularly gynogenesis, are focused on for increasing the ratio of females over males in progeny. Despite the fact that gynogenetic progenies were obtained in many sturgeon species, low survival rates and lack of knowledge about genetic consequences of this treatment, including fitness, hinder the broader introduction of gynogenesis in sturgeon aquaculture. Moreover, difficulties during optimization of sperm DNA inactivation cause variability in results when the proposed protocols are followed.

The present study focused on optimizing chromosomal manipulations in sturgeons, particularly gynogenesis. The reasons of low survival rates were analyzed and the critical steps of gynogenesis induction processes were optimized. In addition, alternative ways of DNA inactivation in sperms were investigated, as well as the influence of native light-dependent DNA repair mechanisms on gynogenesis induction. Methods of interspecific gynogenesis usage for simplifying gynogenetic progeny separation were also proposed.

Spectrophotometry analysis was used to investigate the ability of UV light, as the most common DNA inactivating agent, to penetrate into sperm. In addition, investigation of UV-irradiated sperm motility and results of partial gynogenesis induction showed that low transparency of sperms for UV-light can cause significant heterogeneity of UV-irradiation. As a result, a proper dilution of sperm was suggested as a critical step for homogeneous UV-irradiation of samples. Preliminary investigation of UV absorbance level and effect of UV-irradiation on spermatozoa motility were recommended as the initial steps for appropriate compiling of gynogenesis induction protocols. As a result, UV dose optimization in Siberian sturgeons and sterlet suggests the usage of relatively low UV doses, lying in range from 100 to 200 J/m^2, compared with those reported previously.

Gynogenesis in sterlet was induced with chemical agents that damage sperm DNA, as an alternative to UV irradiation for applied in large-scale production of gynogenotes. All tested substances showed ability to inactivate DNA in spermatozoa, and thus producing gynogenotes. Negative impact of treatments with chemical agents on the sperm motility was observed. Subsequently, these treatments had a low efficiency of gynogenesis induction. The highest percentage of produced gynogenetic larvae 19.8 ± 8.9% was obtained by treatment with aminomethyl-4,5',8-trimethylpsoralen (AMT) at 50 μM followed by UV-A (360 nm) irradiation at dose of 900 J/m^2. Therefore, this treatment could be used as a substitute for commonly used UV-C irradiation, e.g., in the case of large volumes of sperm.

Detailed investigation of photoreactivation in sturgeon sperm revealed a significant level of light-dependent DNA restoration in sperms irradiated with high doses of UV-C light. Induction of gynogenesis with UV-C irradiation followed by exposure to visible light resulted in significant deviations from the typical Hertwig effect. The sensitivity of the comet assay was not sufficient to find significant photoreactivation at low doses of UV-C irradiation. In contrast, the red light with a wavelength of more than 600 nm did not result in decreased DNA damage, instead a moderate increase in damage was observed, i.e., it did not induce photoreactivation. Therefore, the use of infrared light to illuminate work stations during the induction of gynogenesis is suggested.

The use of interspecific gynogenesis, particularly gametes of sturgeon species with different ploidy levels, was suggested as a way to simplify the separation of gynogenotes. In addition, application of this method allowed studying the effectiveness of DNA-inactivation and ploidy restoration treatments separately, as well as evaluation of fitness parameters and survival rates in each group of progeny without the physical separation of fish.

Finally, the protocol for tetraploidization in sterlet was optimized for the prospective using tetraploid individuals for the induction of gynogenesis and androgenesis with diploid eggs and sperm.

In conclusion, the described methods and protocols allowed gynogenesis induction in sturgeons with a survival rate sufficient for aquaculture, taking into consideration their high fertility, although further studies of the consequences of this treatment on fish is required.

CZECH SUMMARY

Optimalizace chromozómových manipulací u jeseterovitých

Ievgen Lebeda

Vysoce výnosný trh s černým kaviárem a decimování divoce žijících populací jeseterů v následku vedou ke zdokonalování akvakultury těchto druhů. Chromozomové manipulace a v jejich rámci obzvláště gynogeneze se nachází v centru pozornosti jako způsob, jak změnit poměr samic a samců v potomstvu. Navzdory faktu, že gynogenetické potomstvo bylo získáno u řady druhů jeseterů, nízká míra přežití a nedostatečná znalost genetických důsledků tohoto ošetření včetně ztráty fitness brání širšímu zavedení gynogeneze v akvakultuře jeseterovitých. Mimoto obtíže s optimalizací inaktivace DNA spermií působí proměnlivost výsledků podle navrhovaných protokolů.

Tato studie byla zaměřena na optimalizaci chromozomových manipulací u jeseterů, a to především na gynogenezi. Byly analyzovány důvody nízké míry přežití a optimalizovány zásadní kroky k indukci gynogeneze. Dále byly prozkoumány alternativní způsoby inaktivace DNA spermatu, stejně jako vliv přirozených mechanismů reparace DNA závislých na světle na výsledky indukce gynogeneze. Byly navrženy metody využití mezidruhové gynogeneze pro zjednodušení separace gynogenetického potomstva.

Spektrofotometrický výzkum byl použit ke zjištění schopnosti UV světla jako nejběžnějšího prostředku inaktivace DNA k proniknutí do vzorku spermatu. Dále výzkum motility ozářených spermií a výsledky parciální indukce gynogeneze ukázaly, že nízká transparentnost spermií pro UV světlo může způsobit významnou heterogenitu UV ozáření. V důsledku toho bylo navrženo vhodné naředění spermií jako zásadní krok pro homogenní UV ozáření vzorku. Výzkum zjištění úrovně UV absorbance a efektu ozáření na motilitu spermií byl doporučen jako první krok pro vhodné sestavování protokolů indukce gynogeneze. Výsledkem je optimalizace dávky záření u jesetera sibiřského a jesetera malého s použitím poměrně nízkých dávek UV pohybujícími se se v rozmezí od 100 do 200 J/m^2, ve srovnání s dříve publikovanými dávkami.

Gynogeneze jesetera malého byla indukována použitím chemických látek, které poškozují DNA spermií jako alternativa k UV ozařování pro účely hromadné produkce gynogenů. Všechny testované substance ukázaly schopnost inaktivovat DNA spermií a produkovat gynogeny. Nicméně byl pozorován negativní dopad ošetření chemickými látkami na motilitu spermií. Následně tyto typy ošetření měly nízkou účinnost indukce gynogeneze. Nejvyšší procento produkovaných gynogenetických larev 19,8 ± 8,9 % bylo získáno při ošetření spermatu aminomethyl-4,5',8-trimethylpsoralenem v koncentraci 50 μM, následovaném ozářením UV-A (360 nm) o dávce 900 J/m^2. Tento typ ošetření by mohl být využíván jako náhrada běžně používaného UV-C záření, například při ošetření velkého objemu spermatu.

Podrobný výzkum fotoreaktivace spermií jeseterů odhalil významnou míru na světle závislé reparace DNA spermií ozářených vysokými dávkami UV-C světla. Indukce gynogeneze s použitím spermií ozářených UV-C zářením a vystavených viditelnému světlu měla za následek významné odchylky od typického Hertwigova efektu. Citlivost kometového testu na detekci poškození DNA nebyla dostatečná k nalezení statisticky významné fotoreaktivace při nízkých dávkách UV-C záření. Naproti tomu červené světlo s vlnovou délkou větší než 600 nm nevedlo ke snížení poškození DNA, místo toho byl nalezen mírný nárůst poškození DNA, tzn. že se neindukovala fotoreaktivace. Proto bylo během indukce gynogeneze navrženo použití infračerveného světla k osvětlení pracovních míst.

Použití mezidruhové gynogeneze, a to zejména využití gamet jeseterovitých druhů s různou úrovní ploidie bylo navrženo jako způsob, jak zjednodušit separaci gynogenů. Kromě toho

aplikace této metody umožňuje odděleně zkoumat efektivnost DNA inaktivace a obnovu ploidní úrovně stejně jako při posouzení fitness parametrů a přežití v každé skupině potomstva bez fyzické separace ryb.

Rovněž byla provedena optimalizace protokolu tetraploidizace jesetera malého pro budoucí uplatnění tetraploidních jedinců s diploidními jikrami a spermiemi při indukci gynogeneze a androgeneze.

Závěrem lze konstatovat, že popsané metody a protokoly dovolují indukovat gynogenezi u jeseterů s dostatečnou mírou přežití pro akvakulturu, s přihlédnutím k jejich vysoké plodnosti, i když je potřeba pokračovat v provádění dalších studií o dalších dopadech tohoto zásahu na rybí organizmus.

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LIST OF PUBLICATIONS

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