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Doctoral thesis

Sperm quality affects the fertilization in freshwater fishes

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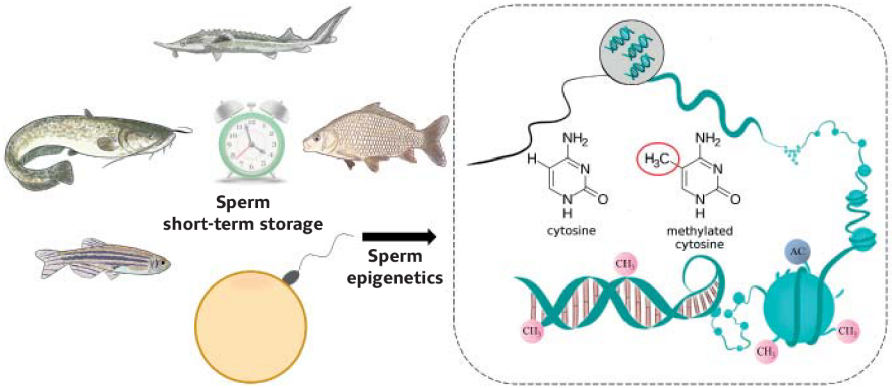

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Fertilization success and offspring performance are highly dependent on the quality of the gametes and fertilization procedures. Usually, in practical applications, short-term sperm storage techniques are used based on demand and its convenience. Phenotypic characteristics of sperm quality (sperm motility kinetics, viability and integrity of the plasma membrane) and its molecular characteristics of DNA fragmentation and sperm epigenetics (such as DNA methylation, histone modifications and non-coding RNA) have been demonstrated to be changed during the storage process (Cabrita et al., 2014; Labbé et al., 2017; Contreras et al., 2020). Therefore, to maintain the genetic diversity and the stability of germplasm resources, optimization and standardization of sperm storage protocol and artificial fertilization with particular consideration to sperm DNA methylation and fertilization capacity assessment are needed in aquaculture practice.

In the present thesis, several experiments were conducted to characterize fish gamete quality including phenotypic changes and molecular modifications during the period of sperm storage. In the first study, an efficient method was established to develop laboratory techniques for the incubation of eggs in sterlet (*Acipenser ruthenus* L.) (**Chapter 2**). Then, a protocol was standardized for artificial fertilization in sterlet (**Chapter 3**). For the next study, sperm quality management in artificial fertilization were studied for selected freshwater species including zebrafish (*Danio rerio* H.), European catfish (*Silurus glanis* L.) and sterlet (**Chapter 4**). Finally, serial experiments were conducted to develop methods for short-term storage of common carp (*Cyprinus carpio* L.) sperm by modifications of both extender and activation solutions that provide practical information for aquaculture practice (**Chapter 5**), and studied aging-related changes in sperm quality with assessment of both phenotypic (sperm motility kinetics) and molecular (DNA methylation) characteristics (**Chapter 6**).

1.2. Model species in the present study

Sterlet

Sturgeons are including 27 species that belong to the order Acipenseriformes, families Acipenseridae (25 species) and Polyodontidae (2 species). They are Chondrostei fishes that stand in an evolutionary position between teleost (bony fishes) and cartilaginous fishes. Sturgeons are known as ''ancient Actinopterygian'' or ''fossil fish'', and they are prized for their meat and caviar (Bemis and Kynard, 1997). However, stocks of sturgeons are dramatically decreasing. Sterlet is a member of Acipenseridae that highly considered as a suitable model species due to its relatively lower age at puberty, short reproductive cycle, and its relatively small size (Billard and Lecointre, 2001). Therefore, it has been a model species not only in biological research due to the evolutionary status of sturgeons, but also in sturgeon aquaculture due to its commercial importance for meat and caviar (Bemis and Kynard, 1997). Sterlet as a sturgeon model species was used to investigate egg incubation techniques, standardize the artificial fertilization procedure, and verify the function of seminal plasma, respectively (**Chapters 2, 3 and 4**).

Zebrafish

Zebrafish is a freshwater fish belonging to the family of Cyprinidae and order of Cypriniformes. It is widely used as a model organism in biomedical research nowadays, and has become a favourite vertebrate species for studies on reproductive biology (Hoo et al., 2016). The advantages of this species as biological model organism include easy and lower cost of

maintenance, rapid growth, short life cycle, annual high fecundity, easy visual observation and manipulation, fast external embryonic development, complete sequenced genome and various strains are available that provide valuable advantages in molecular and genetic research perspectives (Sassen and Koster, 2015; Hoo et al., 2016). Once matured, sperm and eggs are available every day around the year. In the present research, zebrafish was used to develop methods for *in vitro* fertilization with consideration to sperm management (**Chapter 4**).

European catfish

European catfish belongs to the family of Siluridae and order of Siluriformes. It is an important species for aquaculture worldwide, with an annual production in 2018 of *ca*. 6 million (Gisbert et al., 2022). In general, various species of catfishes are economically aquaculture species including *Pangasianodon hypophthalmus* (Pangasiidae), *Clarias gariepinus* (Clariidae), and *Ictalurus punctatus* (Ictaluridae) (Gisbert et al., 2022). Europen catfish is also called wels catfish, sheatfish or just wels, and is native to wide areas of central, southern, and eastern Europe. It usually has a short hatching time (2.5–3 days) and high fecundity. Due to its availability and significant position in aquaculture production, one of the present studies was dealing with the development of protocol for sperm short-term storage with consideration to fertilizing ability of stored sperm in artificial fertilization (**Chapter 4**)**.**

Common carp

The common carp belongs to family Cyprinidae and order of Cypriniformes. It is the most widely used species of cyprinid in aquaculture and the oldest domesticated aquaculture species in the world (Balon, 1995a). There are two main subspecies of common carp: the Asia subspecies (*C. c. haematopterus*) and the European subspecies (*C. c. carpio*) (Balon, 1995b). Cyprinids contribute about 38% of all aquaculture by weight, the common carp accounted for over 7.7 % (>4 million tonnes) of global aquaculture production in 2020 (FAO, 2020). In some European countries, more than 80% of total fish production comes from common carp (Woynarovich et al., 2010). Common carp is also a good model animal to evaluate gamete aging because they display a vast diversity of reproductive modes with a high number of oocytes and spermatozoa. For example, common carp simply produce a huge number of gametes, when one female at the reproduction season is able to provide about 2 kg of eggs (1.6 million eggs) and 20 mL of sperm ($400x10^9$ spermatozoa) from one male during one collection (Linhart et al., 1995a). Due to its sufficient sperm quantity and important status in aquaculture, common carp was used to develop method for artificial fertilization considering sperm management in a large-scale hatchery level and to elucidate epigenetics changes of aging sperm (**Chapter 5 and 6**).

1.3. Incubation of eggs of sterlet

Compared to the fertilized eggs of teleost fishes which are not so adhesive, fertilized eggs of sturgeon become adhesive when faced water. However, eggs adhesive is among key determinant of reproductive success under physiological or hatchery conditions. High mortality caused by anoxia and fungal growth has been reported when eggs were clumped together (Doroshov et al., 1983). Therefore, various incubators including hatchery jars, Zuger incubator or McDonald incubator have been used to incubate fertilized eggs of sturgeons (Mims and Shelton, 2015; Billard, 2000). In many studies, an automatic system with a large volume of circulating water (300 L) has been used to incubate the fertilized sturgeon eggs (Dzyuba et al., 2012; Linhart et al., 2016). Recently, sturgeon eggs have been incubated in Petri dishes with no water flow-through under a constant temperature condition (Fatira et al.,

2018). However, no comparison of efficiency has been made among the methods used for incubation of sturgeon eggs. In research, a convenient incubation technique of fertilized eggs of sturgeons is required to maintain a large number of treatments and replications. Therefore, one of our study was aimed at comparing the efficiency of various methods for egg incubation and embryonic development using sterlet to develop a method for research at hatchery laboratory (**Chapter 2**). This study provides valuable information to develop a simple method for incubation of other fish species in Petri dishes that are reproduced for aquaculture (such as cyprinids, salmonids, catfishes, etc), for biological conservation of endangered species (such as other sturgeon species and eels), and for artificial reproduction of animal models in experimental research (such as zebrafish).

1.4. Artificial fertilization of sterlet oocytes

Sturgeon gametes differ from those teleost fishes due to the presence of multi-micropyle in oocytes and presence of acrosome in sperm (Alavi et al., 2012). Also, sturgeon sperm has the ability to move for several minutes (Linhart et al., 1995b; Psenicka et al., 2011), while duration of sperm motility in most freshwater species lasts for less than 2 minutes with high activity for less than 30 seconds (Alavi et al., 2019). In many teleosts, particularly those with valuable aquaculture practice, the biology of fertilization is well known, and hatchery protocol has been established, for instance common carp (Billard et al., 1995; Linhart et al., 2015) and European catfish (Legendre et al., 1996; Linhart et al., 2004). However, the biology of fertilization in sturgeon eggs is still unknown very well. As above mentioned, unlike those of teleosts, sturgeon eggs possess many micropyle that remain permeable for sperm entry a few minutes after laying (spawning) (Dettlaff et al., 1993). This may suggest that sturgeon oocytes require fewer number of spermatozoa for successful fertilization. Usually, the number of spermatozoa required for successful fertilization is investigated in fertilization *in vitro* and expressed as the sperm/oocyte ratio (Butts et al., 2009; Bokor et al., 2021; Erraud et al., 2022). In this context, the ideal sperm: oocyte ratio for fertilization varies by species (Table 1), and is linked to reproductive physiology, mating strategies, and gamete characteristics (Ginzburg, 1968; Butts et al., 2009; Bokor et al., 2021; Erraud et al., 2022). Noteworthy, when the spermatozoa and egg quantities are consistent in an *in vitro* fertilization, more activation solution can lead to lower spermatozoa concentration surrounding oocytes, which indicates use of large amounts of water influence the fertilization efficiency (Table 1). In addition, some studies reported that large volumes of oocytes with ovarian fluid are capable of changing environmental properties of fertilization medium including changes in magnesium, calcium, and potassium concentrations (Ginzburg, 1968).

In sturgeon aquaculture, the effects of sperm/oocyte ratio, volume of activation medium, and sperm concentration in efficiency of artificial fertilization have not been thoroughly investigated. One of my research was aimed at developing a standard fertilization protocol in sterlet by modifying spermatozoa concentration per mL of activation water and number of spermatozoa per oocyte to identify the optimal volume of activation water and minimum number of spermatozoa for efficient fertilization of oocytes, respectively (**Chapter 3**).

Table 1. *The optimal number of spermatozoa needed to fertilize an egg in fish.*

Fish species	Spz:egg ratio	Egg per trial	AM Volume (mL)	Sperm:egg:AM	Reference
Freshwater fish					
African catfish *Clarias gariepinus*	15,000	1 mL~750	10	1,500	Rurangwa et al. (1998)
Asian catfish *Clarias macrocephalus*	4,000	1 g~470	0.5/g egg	8,000	Tambasen-Cheong et al. (1995)
Brown trout *Salmo trutta*	43,000	nd	nd	nd	Erdahl and Graham (1987)
Tiete tetra *Brycon insignis*	130,870	100g~45,400	100	1,309	Seabra et al. (2022)
Striped catfish *Pangasius hypophthalmus*	189,000	100	nd	nd	Kwantong and Bart (2009)
Channel catfish *Ictalurus punctatus* ♀ × blue catfish *I. furcatus* ♂	5,000–10,000	5 g~250	5	1,000–2,000	Myers et al. (2020)
Common carp *Cyprinus carpio*	20,000	4000	5	4,000	Linhart et al. (2015)
	236,720	5 g~2,930	5	47,344	Linhart et al. (2003b)
Eurasian perch *Perca fluviatilis*	250,000	1 g~566	nd	nd	Bokor et al. (2021)
European catfish *Silurus glanis*	800	6 g~960	6	133	Linhart et al. (2004)
European eel *Anguilla anguilla*	25,000	500	20	1,250	Butts et al. (2014)
Northern pike *Esox lucius*	100,000	0.5 mL~50	1,000	100,000	Cejko et al. (2020)
	26,000	nd	nd	nd	Erdahl and Graham (1987)
	400,000	30 g	15	26,667	Kristan et al. (2020)
Pacu *Piaractus mesopotamicus*	7,000	2 mL~2,700	15–60	117–467	Sanches et al. (2011)
Paddlefish *Polyodon spathula*	1,379,000	4 g~368	nd	nd	Horvath et al. (2010)
Pikeperch *Sander lucioperca*	100,000	200	5	20,000	Kristan et al. (2018)
Rainbow trout *Oncorhynchus mykiss*	100,000–200,000	164–526	nd	nd	Moccia and Munkittrick (1987)
	75,000	1000	nd	nd	Erdahl and Graham (1987)

Table 1. *(continued)*

Freshwater fish					
Saugeye *Sander vitreus* × *S. canadensis*	20,000–100 000	506 g~150,000	1,000	20–100	Blawut et al. (2018)
Sterlet *Acipenser ruthenus*	43,000–430,000	2 g	8	5,375–53,750	Siddique et al. (2015)
Walleye *Sander vitreus*	25,000	nd	nd	nd	Rinchard et al. (2005)
	2,000,000	100	400	5,000	Casselman et al. (2006)
Zebrafish *Danio rerio*	1,333–2,000	20–30	0.36	3,703–5,556	Hagedorn and Carter (2011)
Marine fish					
Atlantic cod *Gadus morhua*	100,000	200	40	2,500	Butts et al. (2009)
Atlantic salmon *Salmo salar*	50,000	300	100	500	Erraud et al. (2022)
Brown-marbled grouper *Epinephelus fuscoguttatus*	1,000	2,000	4	250	Yang et al. (2020)
Meagre *Argyrosomus regius*	150,000	0.5 mL~800	100	1,500	Ramos-Judez et al. (2019)
Senegalese sole *Solea senegalensis*	592 motile spz	0.5 mL~750	5	130	Ramos-Júdez et al. (2021)
Spotted wolffish *Anarhichas minor*	500,000	50	250	2,000	Beirão and Ottesen (2018)
Turbot *Scophthalmus maximus*	6,000	2–38 mL	1–19	316–6,000	Suquet et al. (1995)
	>90,00DF/3,000—40,00WF	5 mL	100	>90DF/30-40WF	Chereguini et al. (1999)
Winter flounder *Pseudopleuronectes americanus*	34,038	0.1 mL~450	40	851	Butts et al. (2012)

Spermatozoa:egg ratio reflects the number of spermatozoa after which adding more spermatozoa to the eggs will not significantly increase fertilization success; Spermatozoa = Spz; AM = activation medium; DF = dry fertilization; WF = wet fertilization; nd: no data

Sperm:egg:AM – calculation according to the ratio of sperm:egg and the volume of activation medium (mL).

1.5. Comparison of sperm short-term storage between artificial and physiological seminal plasma as extenders

To achieve successful artificial reproduction in the hatchery with consideration to creating sufficient genetic variability in offspring, a significant quantity of high-quality gametes is required (Congiu et al., 2011). In most fish species, spermatozoa are immotile in the seminal plasma (Morisawa and Suzuki, 1980). Osmolality and ions (mostly potassium) are the main factors that maintain spermatozoa immotile in the seminal plasma (Billard, 1986). In freshwater fishes including common carp and zebrafish (Poupard et al., 1997; Jing et al., 2009), osmolality is the main factor to maintain sperm in the quiescent state in the seminal plasma, while in salmonids and sturgeons high concentrations of potassium ions in the seminal plasma is the main factor (Cosson and Linhart, 1996; Alavi et al., 2019; Sandoval-Vargas et al., 2021). Consumption of ATP content by spermatozoa is low when they are in a quiescent state which guarantees the spermatozoa still have the energy to become motile after releasing into an aqueous environment (Ingermann, 2008). In freshwater fishes, hypo-osmolality of the aquatic environment activates sperm motility (Morisawa and Suzuki, 1980; Billard, 1986; Alavi et al., 2019). However, in some fish species based on the structure of the urogenital system (urinary bladder and reproductive tract), sperm contamination with urine may occur during the stripping. As a consequence, spontaneous sperm movement occurs during sperm collection. This has been reported in European catfish (Linhart et al., 1987), and various cyprinid species such as common carp (Poupard et al., 1998), zebrafish (Matthews et al., 2018), tench (*Tinca tinca*) (Linhart et al., 2003a) and asp (*Aspius aspius*) (Linhart and Benešovský, 1991). In this case, artificial seminal plasma (also called immobilization solution or extender) plays a crucial role to prevent the spontaneous movement of sperm. Moreover, species-specific artificial seminal plasma has another ability, which is to extend sperm storage time. The usual function of artificial seminal plasma is as follows: 1) improving oxygen supply and exchange by reducing sperm density; 2) maintaining the osmotic and pH equilibrium between the cell and the medium; 3) affecting the maturation of sperm; 4) limiting the activity of seminal plasma lytic enzymes (reviewed by Contreras et al., 2020).

It was reported that low-quality sperm in common carp could be revitalized by incubation in an artificial seminal plasma containing $CaCl_2$, Mg_2SO_4, Tris, NaCl and KCl (310 mOsmol/kg) (Cejko et al., 2019). In addition, it has been demonstrated that testicular sperm from the sterlet will not become motile until it has been mixed with urine, or incubated in seminal plasma or an artificial solution (Dzyuba et al., 2014). Therefore, mixing testicular sperm with urine appears to be critical in the acquisition of potential for motility in sturgeon spermatozoa and fertility maintenance. It is promising to use natural seminal plasma from good-quality sperm to incubate and revitalize low-quality sperm and prolong the *in vitro* storage time.

However, the storage of sperm in an artificial or physiological seminal plasma needs to be evaluated with emphasis on sperm fertility assessment. In the present study (**Chapter 4**), we conducted different experiments on zebrafish, European catfish and sterlet sperm to investigate fertilizing capacity of short-term stored spermatozoa after incubation in artificial or physiological seminal plasma.

1.6. Practical application of activation solutions for fertilization of aged sperm in common carp

In addition to sperm to egg ratio and the number of spermatozoa that affect artificial fertilization, environmental factors such as ion composition, pH and temperature of activation medium, also play important roles in fertilization success (reviewed by Kholodnyy et al., 2020). The optimal activation medium used for artificial fertilization might be species-specific (Viveiros et al., 2019). Sperm motility can be activated with fresh water with osmolality close to zero or solutions containing salts (NaCl, KCl, $NaHCO_3$, $CaCl_2$, $MgSO_4$, Na_2HPO_4, KH_2PO_4) and/or organic compounds (glucose, sucrose, mannitol). Furthermore, several studies have demonstrated the positive effects of ions in activating solutions on sperm motility in cyprinids (Linhart et al., 2003c), salmonids (Dumorne et al., 2018) and sturgeons (Prokopchuk et al., 2016). Most studies have used fresh sperm rather than stored sperm. However, the aged sperm displays different kinetics parameters compared to that of fresh sperm. The present study was aimed at examining the effects of different activation media on fertilizing capacity of aged sperm of common carp for hatchery application (**Chapter 5**).

1.7. Sperm phenotype and molecular changes during short-term storage

Sperm aging refers to the changes in motility and fertilizing capacity that may occur due to age of fish at spawning, due to staying in the reproductive system from the time of release from the testis to spawning (spermiation) or due to storage *in vitro* (Contreras et al., 2020). During the sperm aging induced by short-term storage, sperm changes can be shown in phenotypes and molecular parameters (Figure 1). These parameters indicated whether the sperm quality decreases and probably affects fertilization and future offspring development. Therefore, more detailed information about different parameters of sperm damage during *in vitro* storage is considered in this section.

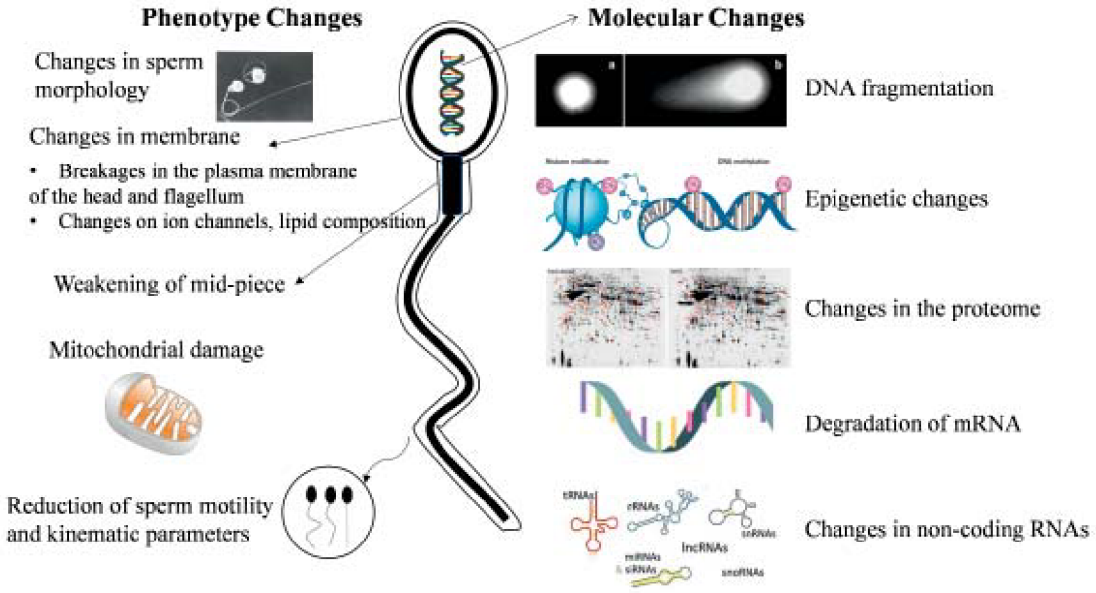

Figure 1. *Main damage inflicted by the process of short-term storage on fish sperm (modification according to Saad et al., 1988; Rajender et al., 2010; Schagdarsurengin et al., 2012; Shaliutina-Kolešová et al., 2020; Dietrich et al., 2021).*

1.7.1. Effects of short-term storage on sperm phenotype

(A) Changes of sperm kinetics parameters: Sperm are remarkably differentiated cells whose primary function is to combine with eggs and fertilize them with a paternal haplotype. Therefore, the movement performance of the spermatozoa is particularly important. A reduction in sperm motility is one of the most evident consequences of fish sperm during cold preservation, yet, not dramatic as cryopreservation especially suitable conditions were applied and have been widely reported in the literatures (Dietrich et al., 2021).

Because the percentage of motile sperm and progressive velocity are predictors of fertility, CASA assessment is of substantial utility in estimating the ability of sperm to accomplish fertilization success (Rurangwa et al., 2001). A strong relationship between sperm motility, fertilization and hatching rates has been reported in fishes including rainbow trout (*Oncorhynchus mykiss*) and seabream (*Pagrus major*) (reviewed by Gallego and Asturiano, 2018), indicating that sperm motility is a key determinant of fertilization. Therefore, it is essential to preserve the sperm movement ability during short-term storage. Moreover, in carp, it has been reported that bad-quality sperm can be revitalised by artificial seminal plasma following 1 h storage in the *in vitro* condition, which is associated with a 3-fold increase in fertilizing capacity (Cejko et al., 2019).

(B) Damage to the sperm plasma membrane: As spermatozoa do not possess an acrosome in most fish species, it might be possible that the plasma membrane is a key component for gamete fusion upon fertilization (Dzyuba and Cosson, 2014). However, the cooling during short-term storage of sperm *in vitro* is a critical stressor that induces increases of oxidative events, moreover, bacterial growth has been observed in this process (Bobe and Labbé, 2009). The properties of fish sperm render them particularly vulnerable to oxidative stress damage, owing to the high presence of PUFAs in the membrane, which makes them an excellent target for Reactive Oxygen Species (ROS) (Cabrita et al., 2014). ROS can attack the sperm plasma membrane and mitochondria, resulting in cell membrane dysfunction. This was confirmed by the presence of plasma membrane proteins both in the extracellular medium and among spermatozoa proteins altered by storage (NAPA in the extracellular medium and SLMAP, GNB1, CANX, ANX in spermatozoa) in common carp sperm (Dietrich et al., 2021).

(C) Reduction of the sperm concentration: Sperm concentration widely varies among fish species. For example, the sperm concentration tends to be $0.5–2.5\times10^{9}$ spz mL^{-1} in sterlet (Shaliutina et al., 2012; Linhart et al., 2020), while higher concentration ($12–21x10^{9}$ spz mL^{-1}) was reported in common carp (Pataki et al., 2022). Frequent studies have indicated that spermatozoa concentration could be affected by fish age, reproductive season, hormonal stimulation methods used and environmental conditions (Alavi et al., 2008; Shaliutina et al., 2012). Few studies reported that short-term storage led to sperm concentration reductions of 26% and 52% after 48 h and 120 h, respectively, in common carp (Dietrich et al., 2021). As sperm concentration plays a decisive role in fertilization success, it is necessary to count sperm numbers prior to artificial fertilization, which determines the milt volume needed to use. Several methods can be used to measure sperm concentration, such as sperm cell counting in a special counting chamber or hemocytometer (e.g. Bürker-Türk, Neubauer, Neubauer improved, Thoma, Makler), or using flow cytometers, automated fluorescent microscopy in the form of nucleocounter devices and Computer-assisted Sperm Analysis (CASA) systems.

(D) Other damages to the sperm phenotypes: Other changes may also occur during sperm storage, such as alterations in sperm morphology and mitochondrial damage, etc. During storage, sperm vitality and normal morphology have been shown to be positively linked with

motility (Fernandez-Novo et al., 2021). Furthermore, it is responsible for higher fertilization ability. It has been demonstrated that sperm of common carp stored with antibiotics (streptomycin and bipenicillin) showed rough head and coiled up flagellum following 6 days, and by increasing the duration of storage to two weeks, the head surface developed verrucose, and the flagellum was coiled around head or itself (Saad et al., 1988). It is worth noting that spermatozoa with coiling in their posterior and middle sections show still motility, but with a slower velocity (Saad et al., 1988). Moreover, cold storage led to the breakages in the plasma membrane of the head and flagellum and weak of the mid-piece with the detachment of flagellum and mitochondrion in Atlantic salmon sperm (Diaz et al., 2019).

Mitochondrial integrity is another important factor in sperm function, and is a parameter to consider when storing spermatozoa. Because spermatozoa are aerobic cells that rely on oxygen to survive, and this reliance is directly related to the amount of oxygen and mitochondrial activity affected during storage. Additionally, the decrease in ATP levels can be blamed for the change and/or damage of mitochondria (Dziewulska et al., 2010). Damage in mitochondrial integrity induced by storage has been reported in many fish species, such as sex-reversed female mandarin fish (*Siniperca chuatsi*) (Liu et al., 2022) and red seabream (*Pagrus major*) (Liu et al., 2015). To supply the protective effect on mitochondrial function, antioxidants were suggested to use during the process of 4 ºC storage (Liu et al., 2015).

1.7.2. Effect of short-term storage on sperm at the molecular level

(A) Nuclear DNA fragmentation or nuclear DNA damage: It is well known that oxidation can compromise DNA integrity in a variety of ways. Strand breaks can occur, and nitrogen bases can be oxidized (Box et al., 2001). Furthermore, the proportion of sperm with fragmented DNA increases with time that can be affected by the techniques of sperm storage (Mohammadi et al., 2019) and fish species (Shaliutina et al., 2013). DNA fragmentation has been attributed to oxidative damage during sperm storage (Li et al., 2010). Significant DNA fragmentation was seen in zebrafish sperm after 12 days of storage in the fish body *in vivo* (Cattelan and Gasparini, 2021). In addition, sperm storage *in vitro* for three-days induced DNA damage in Siberian sturgeon (*Acipenser baerii*) (Shaliutina et al., 2013). A study of sex-reversed rainbow trout suggested that sperm samples with oxidative events treated with 500 mM H_2O_2 can increase the DNA damage level (Pérez-Cerezales et al., 2010). Proteomic results showed that the altered sperm proteins were also involved in DNA repair; 19% of spermatozoa proteins differed in abundance upon sperm storage among cellular localization (Dietrich et al., 2021). However, in Patagonian blenny (*Eleginops maclovinus*), Contreras et al. (2017) found no significant increase of DNA fragmentation neither in undiluted or diluted sperm with 1:1 and 1:3 sperm: diluent stored at 4 ºC for 7 days, which indicated that sperm of this species possesses a high degree of sperm chromatin condensation with proteins, resulting in greater DNA stability.

Sperm DNA quality is a crucial marker of correct genetic material transmission from one generation to the next. During fertilization and embryonic development, sperm DNA fragmentation has been demonstrated to be reversible in oocytes and early embryos (Herráez et al., 2017). Several repair mechanisms can be employed after fertilization such as base excision repair for the single-strand breaks, homologous recombination and non-homologous end joining to repair DNA double-strand breaks (review by Herráez et al., 2017). Inhibition treatment of sperm DNA repairment with 3-aminobenzamide (base excision repair pathway) showed that during the first cleavage, the oocyte partially repairs the damage; despite DNA damage, trout sperm retains its capacity to fertilize, but embryo survival decreases (Pérez-Cerezales et al., 2010). Similar results were found in a study on zebrafish; although

sperm DNA fragmentation resulting from increased incubation time did not affect oocyte fertilization capacity, it has a major impact on the normal development of later embryos and overall reproductive success (Gosálvez et al., 2014). Therefore, DNA fragmentation in aging spermatozoa after fridge storage may decrease embryo survival and influence development but proclaim a slight effect on fertilization capacity.

(B) Introduction to epigenetics: It is essential to briefly review epigenetic mechanisms in cells before delving into the short-term-storage-induced epigenetic modifications in sperm.

The inheritable changes in gene expression due to mechanisms other than modifications in the DNA sequence are referred to as epigenetics. To initiate and maintain epigenetic silencing, two main categories of DNA silencing methods are usually tested: DNA methylation and histone modification (Vaissière et al., 2008).

***DNA methylation*:** It has been assumed that animal phenotypes were a functional consequence of the arrangement of their nucleotide bases. However, a question remained why does a specific cell type have a unique collection of activated genes, while all of the body cells have the same DNA? It is caused by gene regulation. Data from organisms has shown the importance of other genomic modifications that cause an individual's phenotype. These changes, known as "epigenetics", may persist during mitosis and meiosis, and have an impact on transcriptional control of a gene in many somatic cell types as well as germ cells (Feng and Chen, 2015). In this context, DNA methylation is one of the most studied and crucial epigenetic processes.

DNA methylation is a major epigenetic modification by which methyl groups are added to a DNA base, called the "fifth nucleotide". In vertebrates, this modification mostly occurs on the cytosine base, and only when this cytosine is followed by a guanine (Figure 2), resulting in the CpG dinucleotide's methylation status. The CpG sites occur with high frequency in genomic regions called CpG islands (or CG islands). The CpG islands are not randomly distributed, but are concentrated upstream of the corresponding genes, for instance in the promoter region.

Usually, it has been shown that cytosine DNA methylation in specific genomic loci is linked to silent chromatin and further takes part in regulating gene expression. In zebrafish, the same relationship between DNA methylation and suppression of gene expression has been observed, because the most developmental stage-specific differentially methylated regions (dsDMRs) with decreasing DNA methylation levels during embryogenesis are located in developmental enhancers (Lee et al., 2015). Methylation represses transcription either directly by inhibiting transcription factor binding to DNA or indirectly by methyl-CpG-binding proteins recognizing methylation sites and recruiting co-repressors (Watt and Molloy, 1988). However, it is not always linked to activating transcription since transcription requires the activation of the regulatory network.

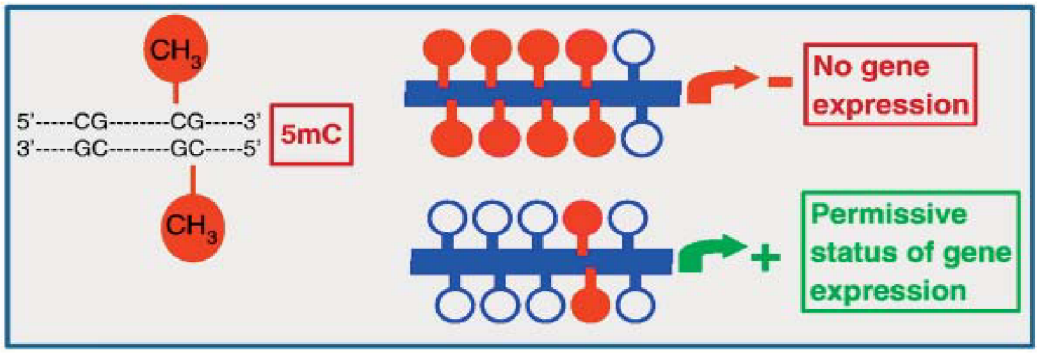

Figure 2. *A schematic of DNA methylation. Hyper or hypo-methylation of the gene promoter region signs a repressive or permissive status of transcription (Labbé et al., 2017).*

DNA methylation includes *de novo* methylation, which needs DNA methyl transferase (DNMT). The DNMT3a, DNMT3b and DNMT3L are involved in DNA methylation, while DNMT1 maintain methylation. During normal DNA replication, the DNA methylation pattern is passed down to the daughter cells at each cell division (cellular memory). DNMT 1, which recognizes hemi-methylated DNA, catalyses cytosine methylation of the newly generated strand after DNA methylation. DNMT1, therefore, inhibits 5-methylcytosine (5mC) maintenance upon 5-hydroxymethylcytosine (5hmC) formation.

During mammalian early development, the level of DNA methylation changes throughout the genome, directing pluripotent stem cells to differentiate into discrete lineages that give rise to different tissue types. Active DNA demethylation refers to an enzymatic process that removes or modifies the methylation group from 5mC. During the phylotypic phase, active DNA demethylation has been seen, for instance, in the enhancer regions of developmental genes (Bogdanović et al., 2016).

Apart from DNA methylation, at the same time, demethylation also plays a crucial role in the biological process. Active and passive demethylations are two main types of demethylation. The active demethylation pathway is thought to involve the ten-eleven translocation (TET) enzymes (TET1, TET2 and TET3). They are dioxygenases that are reliant on 2-oxoglutarate (2-OG) and Fe(ii), iteratively oxidizing 5mC to 5hmC which is the first oxidative product in the active demethylation of 5mC, then 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). Under the function of thymine DNA glycosylase (TDG) mediation, base excision repair (BER), then removes 5fC or 5caC and finally replaces it with unmodified cytosine (Kohli and Zhang, 2013). Passive demethylation is another process to lead the methylation into demethylation. The loss of 5mC throughout successive rounds of replication in the absence of functional DNA methylation maintenance machinery is referred to as passive DNA demethylation. Through this process, DNMT1 is inhibited, and the newly synthesized strand is not remethylated (Hahn et al., 2014; Figure 3).

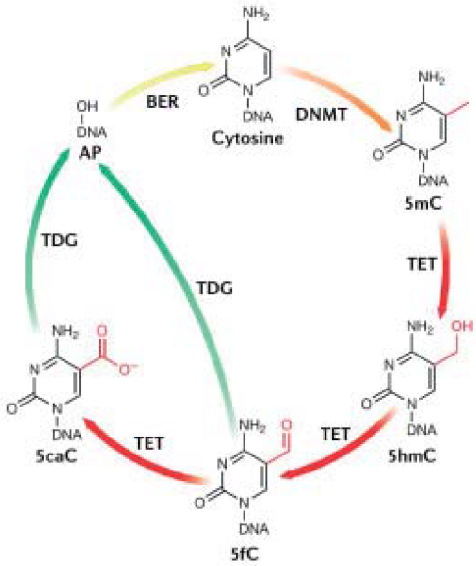

Figure 3. *The active demethylation pathway. In eukaryotes, methylation at the C5 position of cytosine (C) is catalysed by DNA methyltransferases (DNMTs), which use S-adenosylmethionine (SAM) as the methyl donor (not shown), resulting in the formation of 5-methylcytosine (5mC; orange arrow). The postulated active demethylation pathway involves the iterative oxidation of 5mC to 5-(hydroxymethyl) cytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) by ten-eleven translocation (TET) enzymes (red arrows). 5fC and 5caC then can be excised by G/T-mismatch-specific thymine DNA glycosylase (TDG; green arrows) to form an apyrimidinic (AP) site, which further undergoes base excision repair (BER; yellow arrow) to reinstall the cytosine. Structural modifications at each transition are shown in red (reviewed by Raiber et al., 2017).*

***Histone modification*:** Histones are highly basic proteins with a lot of lysine and arginine residues that are found in the nucleus of eukaryotic cells. They function as spools around which DNA winds to form nucleosomes, which are structural units. The chromatin structure is usually comprised of four core histones (H2A, H2B, H3 and H4), the linker histone H1. Furthermore, multiple enzymatic systems ensure the formation and maintenance of epigenetic patterns, including histone acetyltransferases (HATs) and histone deacetylases (HDACs). The activity of these enzymes maintains a balance between acetylation and deacetylation changes (reviewed by Marmorstein and Trievel, 2009).

(C) Changes in sperm DNA methylation: The primordial germ cells (PGCs) in the embryo undergo a complete rapid global demethylation to remove parental imprints. Then, upon spermatogenesis, new epigenetic marks are established for gender-specific imprints in relation to sperm function to ensure appropriate embryo development after fertilization. However, DNA methylation is maintained during spermatogenesis from the spermatocyte to the mature sperm (Nevin and Carroll, 2015).

Usually, the sperm methylome is significantly hypermethylated compared to the oocyte in mammals and zebrafish (Jiang et al., 2013; McCarrey, 2014). In zebrafish sperm genomes, more than 90% of CpG content is methylated, while oocyte genomes have 80% of methylated CpG (Jiang et al., 2013). In human sperm, the sperm genome contains only a few hypomethylated CpGs, which are found in poised promoters and imprinted sites of developmentally essential genes (Hammoud et al., 2009).

In fish sperm, the global DNA methylation level remained relatively constant over time post hormonal induction of sperm maturation, as well as during the reproductive season (Depincé et al., 2020). Cryopreservation of zebrafish spermatozoa with methanol increased global DNA methylation but it was without effects on goldfish spermatozoa (Depincé et al., 2020) that may represent species-specificity. Herranz-Jusdado et al. (2019) reported that hypomethylation of European eel (*Anguilla anguilla*) sperm DNA was induced by DMSO based protocol rather than methanol. To be noticed, there is a lack of a better characterization of molecular analysis in sperm subpopulation (Depincé et al., 2020), however, motility-dependent spermatozoa subpopulations may have the ability to be involved in the offspring and further influence population phenotypes. It has been suggested that genes of WDR3/UTP12 and GPCR families are determinants of fertility performance in striped bass (*Morone saxatilis*), a total of 171 differentially methylated regions are correlated to fertility (Woods et al., 2018). These studies suggest that sperm DNA modification is critical for sperm fertilizing ability, is a species-specific character, and is affected by cryopreservation. In the latter case, cryoprotectant may play a critical role in the level of sperm DNA methylation following cryopreservation. Therefore, DNA methylation analysis becomes an effective tool for male fertility assessment to identify those with superior gametes to improve reproductive efficiency in aquaculture practice. However, the identification and characterization of DNA methylation in aging fish spermatozoa following short-term storage has not been elucidated. In the present study, experiments were conducted to investigate the effect of short-term *in vitro* storage on sperm phenotypes, as well as spermatozoa DNA methylation in common carp (Chapter 6).

(D) Embryo DNA methylation reprogramming: DNA methylation has an important role in embryonic development after sperm-egg communication upon fertilization. After fertilization, in transcriptionally quiescent cleavage embryos of zebrafish, DNA methylation patterns are programmed; paternally inherited patterns are maintained, but maternal patterns are reprogrammed to match the paternal patterns (Murphy et al., 2018). Zebrafish sperm and oocytes lack DNA methylation at the promoters of housekeeping genes and ‘‘Placeholder’’

nucleosomes (containing histone H2A variant H2A.Z(FV) and H3K4me1) maintenance in these regions; meanwhile, sperm lack DNA methylation at several hundred loci important for early development, however oocytes possess it. In this case, during embryo development, the placeholder starts to establish and leads its methylation lever to the same as sperm during the stage of zygotic genome activation. Additional gene classes are linked to the late development, DNA methylation presence in sperm rather than oocyte, therefore, placeholder antagonism (i.e., H2AFV is removed) in eggs and DNA becomes methylated (Murphy et al., 2018).

Overall, it is well known in zebrafish that offsprings inherit the DNA methylome from parents, prior to the 16-cells stage, maternal and paternal DNA methylation patterns are maintained; However, subsequently, from 32 cells onward, the embryo is reprogrammed to a pattern similar to that of the sperm methylome at the mid-blastula stage (MBT) (Jiang et al., 2013; Figure 4). At MBT, it is also the key stage that large-scale embryonic transcription begins. The other embryonic events, such as alterations in the cell cycle, chromatin state and ratios of nuclear and cytoplasmic components are mechanistically coordinated with zygotic genome activation (ZGA) (Jukam et al., 2017). In this regard, studies on sperm DNA methylation are particularly important as epigenetic programming in male gametes, as well as female gamete, is crucial for post-fertilization success and healthy offspring development (Boissonnas et al., 2013). In aquaculture, due to the demand for artificial fertilization and genetic breeding, it is worth investigating the transmission of fish sperm DNA methylation inherited into the embryos and larvae in further studies.

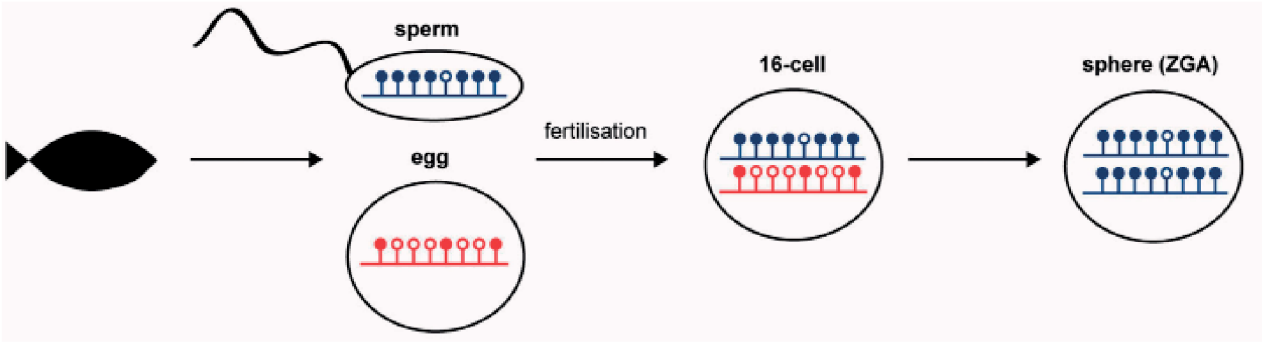

Figure 4. *DNA methylation remodelling during zebrafish (Danio rerio) fertilization and embryogenesis. In the zebrafish embryo, the maternal genomic contribution becomes extensively remodelled to equal the paternal one at the onset of zygotic genome activation (ZGA). This process includes both de novo methylation of oocyte hypomethylated regions and demethylation of oocyte-specific differentially methylated regions (Bogdanović et al., 2014).*

(E) Sperm histone modification: Compared to normozoospermic spermatozoa, sperm samples with combined and isolated abnormalities of total motility, progressive motility, and morphology have shown different and changed post-translational modification of histones (PTMs) (Schon et al., 2019). Dietrich et al. (2021) reported that proteins changes of common carp sperm following storage involved in histone binding protein (RBBP7) and HINT1, which may be attributed to histone modifications as the histone chaperones RBBP7 and HINT1 are integral subunits of protein complexes involved in acetylation/deacetylation of histone proteins (Murzina et al., 2008).

(F) *Other molecular changes* in sperm: Many other molecular changes are involved as the markers of sperm quality, such as proteome, transcripts and non-coding RNAs (ncRNAs).

There are many other molecular markers for sperm that may change during short-term storage such as proteomes, transcriptomes and non-coding RNAs (ncRNAs).

Proteomics is a biochemistry tool that identifies hundreds to thousands of proteins (Ciereszko et al., 2017). Using this molecular approach, we can identify a number of uncharacteristic proteins in fish sperm (Dietrich et al., 2014a,b; Nynca et al., 2014) and search for potential physiological correlations with phenotypic changes, viability and fertility of during storage. In common carp, proteomic analysis has been revealed that the majority of the changed sperm proteins following short-term storage are related to energy metabolism, flagellar functions, stress response, posttranslational modification and protein folding (Dietrich et al., 2021). The authors also found alternations in protein expression that were associated with signal transduction and DNA repair. However, it has been also suggested to detect the proteins in the fluid surrounding extended sperm rather than spermatozoa due to the low protein concentration (1–2 mg mL^{-1}) in fish seminal plasma (Ciereszko et al., 2011; Dietrich et al., 2021). Overall, proteomic research is crucial for optimizing protocols for sperm short-term storage to develop artificial reproduction in fish.

In addition, it is generally well known that spermatozoa are transcriptionally inactive cells, and for many years it has been considered that the RNA population of spermatozoa is non-functional since these molecules are left over from spermatogenesis (Hosken and Hodgson, 2014). However, it is now widely known that spermatozoa deliver more than only the paternal genome to the oocyte, and that remaining spermatogenesis mRNAs can play important roles in early embryonic development and fertilization success (Lalancette et al., 2008; García-Herrero et al., 2011). With the prolonged fish sperm storage *in vitro*, it has been observed that the RNA quantity was gradually decreased both in the cytoplasm and in the mitochondria of the spermatozoon; the transcripts that reduced were mostly associated with energy metabolism and stress response (Yang et al., 2021).

Only 2.5% of the total genome is made up of the coding region of DNA. Despite this, the majority of the genome (85%) can be transcribed as non-coding RNA (ncRNA). Housekeeping RNA, such as transfer RNA (tRNA), ribosomal RNA and regulatory ncRNAs, which have a variety of roles, including post-transcriptional regulation of mRNA, are the two categories of ncRNAs (Le Blévec et al., 2020). In addition, long non-coding RNAs (lncRNAs) can influence gene expression of epigenetic regulation by functioning as guide molecules for chromatin-modifying protein complexes (Hombach and Kretz, 2016). In frozen sperm, the non-coding RNAs such as microRNA and piwi-interacting RNA (piRNA) was altered by freezing process. It has been observed that 83 miRNAs and 79 putative piRNAs clusters are differentially expressed in high-motile and low-motile sperm fractions (Capra et al., 2017). Based on these alternations, the authors suggested functional changes in sperm and increase in germ cell apoptosis particularly in the low motile fraction. Similar sperm motility reduction was observed after short-term storage even not dramatic changes as in cryopreservation (Dietrich et al., 2021). Therefore, further studies need to characterize ncRNA function in fish sperm during short-term storage.

1.8. Objectives of the thesis

The main goal of the present study was to optimize protocols for sperm management and artificial fertilization of selected freshwater fishes with top concerns in aquaculture practice (common carp, European catfish and sturgeon) or in biomedical research (zebrafish). To approach the goal, series of experiments have been conducted during 4 years of my Ph.D. work to optimize methods for egg incubation (**Chapter 2**), artificial fertilization of eggs (**Chapter 3, 4, 5 and 6**), and short-term storage of sperm (**Chapter 4, 5 and 6**). To better understand the effects of short-term storage on sperm functions, the motility kinetics and fertilizing ability, epigenetic changes during the period of storage were analysed with consideration to DNA

methylation levels (**Chapter 6**). Our results provide us with new insights into reproductive biology, and to develop methods for artificial reproduction of fishes in aquaculture and biological models in biomedicine.

The present dissertation contains the following studies:

Chapter 2: Optimization of sterlet (*Acipenser ruthenus* L.) egg incubation

Chapter 3: Standardization of egg activation and fertilization in sterlet (*Acipenser ruthenus* L.)

Chapter 4: Sperm management and fertilization of zebrafish (*Danio rerio* H.), European catfish (*Silurus glanis* L.) and sterlet (*Acipenser ruthenus* L.)

Chapter 5: Practical use of extender and activation solutions for short-term storage of common carp (*Cyprinus carpio* L.) milt in a hatchery

Chapter 6: Changes in phenotypes and DNA methylation of *in vitro* aging sperm in common carp, *Cyprinus carpio* L.

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CHAPTER 2

OPTIMIZATION OF STERLET (*Acipenser ruthenus* L.) EGG INCUBATION

Cheng, Y., Xin, M.M., Gela, D., Rodina, M., Tučková, V., Kašpar, V., Siddique, M.A.M., Shelton, W.L., Linhart, O., 2020. Optimization of sterlet (*Acipenser ruthenus*) egg incubation. Animal Reproduction Science 215, 106334.

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Optimization of sterlet (*Acipenser ruthenus*) egg incubation

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ABSTRACT

Sterlet *Acipenser ruthenus* was used to assess egg and embryo development when incubated at 17 °C in Petri dishes placed in a hatchery tank (300 L recirculating dechlorinated water) with incubation occurring in a static tabletop system in an air-conditioned laboratory, or in a 700 L Q-cell incubator. Eggs in each dish were placed in a plastic box with 300 mL dechlorinated water. Separated eggs from three individual females were fertilized using pooled sperm from four males with there being four replicates. There were no differences ($P > 0.05$) in mean percentages of neurulation and embryos undergoing cleavage for eggs incubated in the hatchery tank and with use of the static tabletop system. Furthermore, there were no differences ($P > 0.05$) in percentage of embryos undergoing cleavage, neurulation and hatching for each female when eggs were incubated using the two systems. Results indicate a Petri dish placed in a small plastic box with 300 mL of dechlorinated water was adequate for incubation of sterlet eggs. Results of the study also indicate that with the static system: 1) eggs should be fertilized from each female to retain individual identity; 2) eggs should be dispersed in Petri dishes to avoid clumping; 3) water should be changed at 24 h, but not at 48 h (neurulation) post-fertilization; and 4) embryos that do not optimally develop should be removed the day after neurulation (72 h of post-fertilization period) and water should be exchanged every day subsequent to the 48 h time-point post-fertilization.

1. Introduction

Sturgeon aquaculture has been expanding worldwide because of international demand for flesh and caviar for human diets, and conservation programs for sustaining the species (Bronzi et al., 2011). Understanding reproductive biology and optimizing methods for the artificial propagation of sturgeon are important for development of controlled reproduction protocols when sturgeons are in maintained in captive environments and to increase the efficacy of aquaculture programs (Alavi et al., 2008). With sturgeon, eggs are incubated in Zuger or McDonald's incubation bottles (Billard, 2000; Mims and Shelton, 2015) using specialized incubation equipment (Dettlaff et al., 1993; Chebanov and Billard, 2001).

This incubation equipment has been used for hatching large volumes of eggs usually in upwelling (increasing water surface level)

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flow-through systems. A simple but efficient circulation technique would facilitate the production of fertilized eggs for research purposes. In several studies, there has been use of systems which require a large volume of circulating water (~300 L) to incubate and hatch the fertilized sturgeon eggs (Dzyuba et al., 2012; Linhart et al., 2016; Xin et al., 2018). Recently Petri dishes without water flow-through occurring in which there were constant temperature conditions imposed have been used to incubate sturgeon eggs (Fatira et al., 2018). None of these systems have been compared for efficacy of sturgeon production. The objective of this study, therefore, was to compare the incubation of sterlet *Acipenser ruthenus* eggs and embryonic development at 17 °C in Petri dishes using a static condition with the efficacy when using a flow-through system for egg incubations and embryonic development. The current study is the first in which there was use of a novel technique to incubate sterlet and other sturgeon eggs for research purposes, in which multiple treatments can easily occur.

2. Materials and methods

Experiments with fish were specifically approved by the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany, based on the EU-harmonized Animal Welfare Act of the Czech Republic.

2.1. Broodstock husbandry and gamete collection

Sterlet males weighing 0.8–1.9 kg and females weighing 1.5–2 kg were obtained from the experimental station of the Faculty of Fisheries and Protection of Waters, the University of South Bohemia at Vodnany, Czech Republic. Prior to experimentation, male and female broodstock were maintained in separate 4 m^3 hatchery tanks with a water flow rate of 0.2/L, O_2 of 7.0 mg/L and a water temperature of 15 °C.

Twelve females (six per experiment, total of two experiments) were used in the study. Ovulation was induced using carp pituitary extract (CPE) dissolved in 0.9 % (w/v) NaCl solution; 0.5 mg/kg body weight was injected as a priming dose and a second injection of 4.5 mg/kg body weight was administered 12 h later. Eggs were collected 30 h after the second injection. Eggs were stripped being careful when conducting the standard laboratory procedures so as to not injure the specimen from which eggs were collected and to ensure eggs were not contaminated by body fluids. Eggs were subsequently placed in plastic bowls. The eggs considered to be of greatest quality from three individual females per experiment were visually selected and used within 1 h of collection for each fertilization assay. The criteria for selection of eggs was those with a darker coloration (i.e., the darker the more desirable) and when there was an absence of eggs undergoing decomposition.

A single intramuscular injection of carp pituitary extract was administered to each of 16 males (eight males for each experiment, total of two experiments) at a dose of 4 mg/kg body weight. A plastic catheter (4 mm diameter) was used to collect milt 48 h after the injection and milt was stored at 4 °C. The sperm motility was evaluated using a microscope and semen from four males per experiment with a minimal requirement of > 80 % motility was pooled in equal proportions. Prior to fertilization, the concentration of pooled sperm was determined using a Bürker cell hemocytometer (Marienfeld, Germany, 12 squares counted for each male) with utilization of an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) and expressed in 10^9/mL.

2.2. First experiment

In the first experiment, the development of the eggs and embryos was compared when these were contained in Petri dishes in a static tabletop system in a controlled air-conditioned laboratory and in a hatchery incubation tank at 17 °C.

2.2.1. Fertilization

For the fertilization assay, eggs from individual females (0.6 g, ~50 ova) were placed in a 25 mL beaker on a shaking table and there was insemination using 5 µL of sperm (1,500,000 spermatozoa) utilizing a pipette for transfer, which was inserted into the bottom of the beaker near the ova. This procedure was repeated four times, resulting in four beakers being placed on the shaking table (at 17 °C). The shaking table was activated for 2 min (150 rpm) and 3 mL of water was pipetted into each beaker during this period. The fertilized eggs were distributed taking great care in conducting the transfers from the four beakers into four glass Petri dishes (95 mL; 9 cm in diameter and 1.5 cm in depth; Fig.1). Each Petri dish was filled with dechlorinated water.

2.2.2. Incubation in an air-conditioned laboratory

Each Petri dish was placed in a small plastic box (13.5 cm × 10 cm × 6.5 cm) which contained dechlorinated water at a volume (300 mL) that resulted in the box being filled to one third of its total capacity. The four small boxes were placed in one large plastic box (28 cm × 21.5 cm × 7.5 cm; Fig. 1). The air temperature was maintained at 17 °C. The dechlorinated water was carefully replaced in the small plastic boxes after 24 h; and the experiment was completed when the process of neurulation occurred (72 h post-fertilization). The stages of embryonic development were determined by images recording of embryos using a digital camera (Olympus Corp., Tokyo, Japan, model E-M5MarkII) at 5 h (at 4 cell developmental stage; Fig. 2) and 72 h post-fertilization (Fig. 3). Only viable eggs that had developed > 4 cells or to the neurula stage were used for data analyses.

2.2.3. Incubation in a flow-through hatchery tank

Fertilized eggs attached to the Petri dishes were gently submerged in a small plastic box (19 cm × 4.5 cm × 15 cm) which had a

Fig. 1. Large plastic box containing four smaller boxes (each with 300 mL dechlorinated water) with Petri dishes being located in the smaller boxes that contain fertilized sterlet eggs incubated at 17 °C.

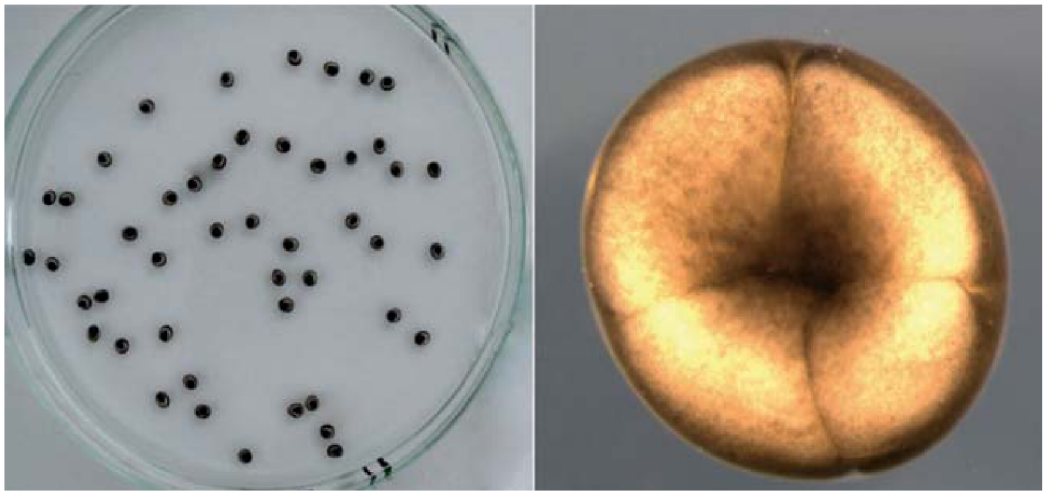

Fig. 2. Petri dish containing sterlet embryos at the 4 cells cleavage stage (left picture) and embryos observed at greater magnification for developmental assessments (right picture) recorded 5 h after activation and initiation of incubation at 17 °C.

permeable screen at the bottom and water was gently sprayed at the water surface (Fig. 4). All these plastic boxes were attached to a water tank (300 L volume) with a continuous recirculation of water that was treated using a germicidal UV light. The water temperature of the tank was maintained at 17 °C with recirculation occurring, however, the water was not replaced during the experiment.

2.3. Second experiment

In the second experiment, incubation of eggs was continued until there was larval development. The stages of egg and embryo developments while using the tabletop static Petri dish system in the controlled a

ir-conditioned laboratory was compared with those when there was incubation in a special large thermostatic incubator box (Q-

Fig. 3. Petri dish containing sterlet embryos at the neurulation stage of development (left picture) and embryos observed at a greater magnification for developmental assessments (right picture) recoded 72 h after activation and initiation of incubation at 17 °C.

Fig. 4. Experimental incubation tank with 300 L re-circulated water and small boxes into which Petri dishes containing sterlet eggs were placed and incubated at 17 °C.

cell 700 inox+, Pol-Lab, Poland; www.pol-lab.eu/en/oferta,q-cell_series_incubators13.html) at 17 °C.

Egg development was recorded twice as in the second experiment, however, unlike the first experiment, incubation was continued until the time of hatching at 144 h post-activation. Hatched larvae were manually counted immediately after hatching and compared

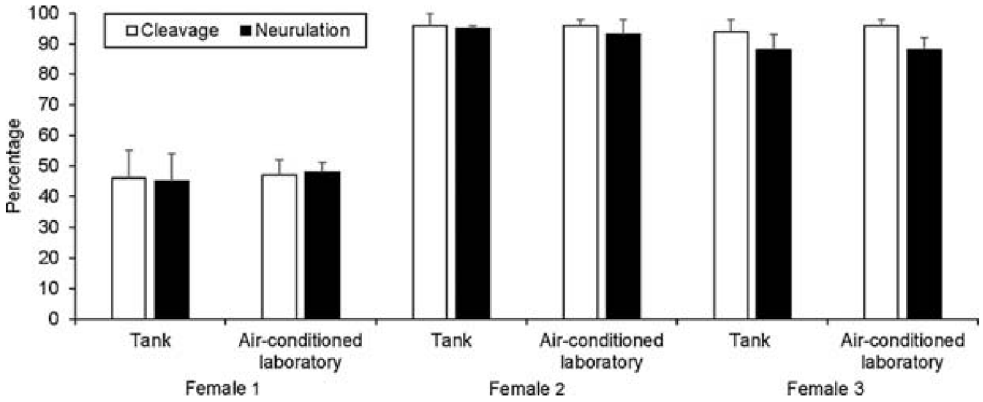

Fig. 5. Percentage cleavage and neurulation rates (%) of embryos from three sterlet females incubated in Petri dishes when there was use of a static tabletop system in a controlled air-conditioned laboratory or hatchery incubation tank at 17 °C; Mean values for four replicates ($n = 4$) are shown; vertical lines are S.D.; Percentages for cleavage and neurulation rates were not different for each female ($P > 0.05$).

with the initial number of eggs in the corresponding Petri dish. Dechlorinated water was changed taking great care to not disrupt egg and embryonic developments in the small plastic boxes at 24 h post-fertilization and non-viable embryos were removed; water was exchanged daily after neurulation (72 h post-fertilization) until that time of hatching.

2.4. Statistical analysis

The non-parametric Wilcoxon signed-ranks test for paired data was used to determine the differences in percentages of cleavage, neurulation, and hatching for each female when there was incubation in the hatchery tank and air-conditioned laboratory and when there was incubation in the hatchery tank and Q-cell incubator box. For all analyses that were performed there were considered to be differences when there was a $P < 0.05$ by using SPSS 20.0 for Windows.

3. Results

3.1. Experiment 1

There was successful incubation of eggs using the tabletop static Petri dish system and the hatchery tank system. There were no differences in the percentages for cleavage or neurulation occurring for embryos incubated in the Petri dishes using the static tabletop system in the controlled air-conditioned laboratory and the hatchery incubation tank at 17 °C ($P > 0.05$; Fig. 5). Percentages of eggs in which cleavage occurred from Females 2 and 3 that were incubated in the hatchery tank (96 % and 94 %, respectively) and in the air-conditioned laboratory (96 % for both females) were comparable. The percentage of eggs undergoing cleavage for Female 1 was less in comparison to eggs from Females 2 and 3, however, there was a similar percentage of eggs undergoing cleavage when there was incubation in the hatchery tank (46 %) and in the air-conditioned laboratory (47 %). In general, the percentage of neurulation of embryos that developed from eggs of all three females had similar trends as occurred with the percentage of eggs undergoing cleavage, indicating the incubation conditions were efficacious for sturgeon experiment.

3.2. Experiment 2

The fertilization rate of eggs in Petri dishes was similar with use of the tabletop system controlled in the air-conditioned laboratory and in the special Q-cell incubator box. There were no differences in percentage of embryos undergoing cleavage, neurulation, and hatching when there was use of the two incubation methods ($P > 0.05$; Fig. 6). Percentages of embryos undergoing cleavage and neurulation that resulted from fertilization of eggs from Females 1 and 2 were similar when there was use of the Q-cell incubator box (> 88 %) and tabletop system (> 86 %). The percentage of eggs undergoing cleavage and embryos developing to the neurulation stage as a result of fertilization of eggs of Female 3 was less than those from Female 1 and 2, however, was similar to that for eggs incubated in the Q-cell incubator box (> 78 %) and in the air-conditioned laboratory (> 86 %). The percentage hatching of eggs from Female 2 that were incubated in the Q-cell incubator box (87 %) was similar to that of eggs incubated in the air-conditioned laboratory (93 %). Eggs of Females 1 and 3 incubated in the air-conditioned laboratory had greater hatching rates (83 % - Female 1 and 80 % - Female 3) in comparison to those incubated in the Q-cell box (72 % - Female 1 and 76 % - Female 3).

4. Discussion

Fertilization and hatching rates were comparable with use of the incubation systems utilized in the present study. This finding indicates the environmental conditions of the 300 mL small plastic boxes that were used for incubations in the static tabletop system

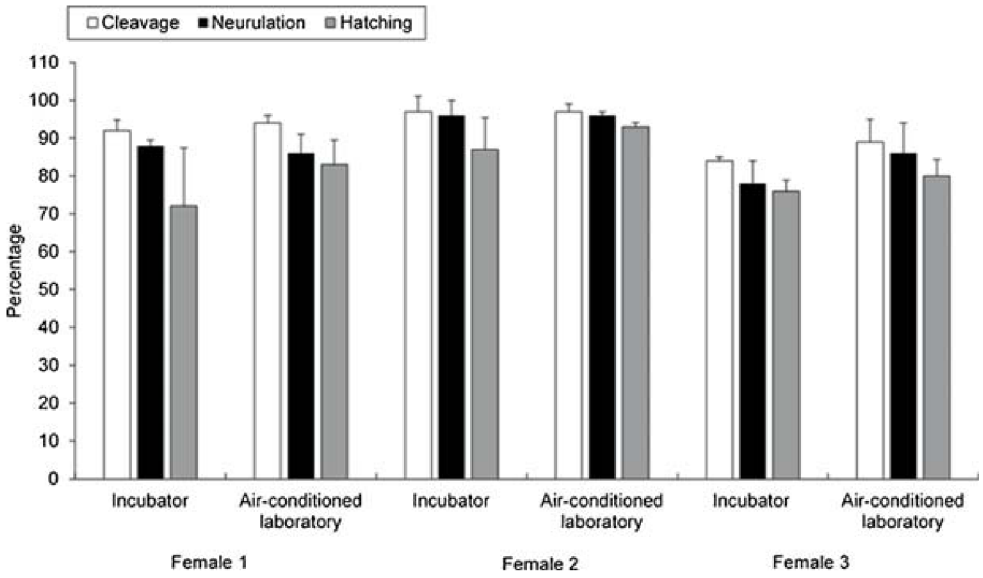

Fig. 6. Percentage cleavage, neurulation and hatching rates (%) of embryos and larvae from three sterlet females when eggs were incubated in static Petri dishes using a tabletop system in a controlled air-conditioned laboratory or in a large thermostatic incubator box (Q-cell 700 inox+) at 17 °C; Mean values of four replicates ($n = 4$) are shown; vertical lines are S.D.; Percentage cleavage, neurulation and hatching rates were not different for any of the females ($P > 0.05$).

in an air-conditioned laboratory were sufficient for the optimal development of sterlet embryos.

During the present study, there was an attempt to standardize procedures and techniques. The use of eggs pooled from different females would have avoided the effect of differences in the females on the results (Dzyuba et al., 2012; Xin et al., 2018). In the present study, however, eggs from different females were not pooled and the inter-individual difference in quality of eggs was clearly evident. In general, sturgeon eggs have a relatively greater viscosity than eggs of many other fish species making subsampling difficult. If eggs from several females are pooled into one sample, the variability of results could be great enough that this variation could mask any differences resulting from treatments. This occurred in some previous studies with sturgeon where the results were highly variable which complicated data interpretations (Dzyuba et al., 2012; Xin et al., 2018).

In general, a pool of sperm was used to allow homogeneous fertilization by reducing the problems of interaction between sperm and eggs (Linhart et al., 2005). The amount of sperm in the 3 mL of water during activation (1,500,000 spermatozoa) was optimal for limiting polyspermy (Iegorova et al., 2018) and for obtaining a highly acceptable fertilization rate. The dilution of sperm at rations of 1:100 to 1:200 with water during egg fertilization is a procedure used to reduce the occurrence of polyspermy (Dettlaff et al., 1993). With the conditions in the present study, there was a much greater ratio of 1:600 utilized. This means that the range used in present study was similar to what occurs with sturgeon in their natural habitat.

During the fertilization assay, milt was not placed directly on the eggs, but rather the pipette was inserted to the bottom of the beaker next to the eggs and water was added.to activate the gametes. Homogeneity of the sperm in the water, therefore, was achieved from the beginning of the fertilization process. It was also important to carefully mix the eggs with milt and activation media when these were located on a shaker table at a setting of 150 rpm to ensure greater homogeneity and to reduce adherence of eggs to each other.

After activation, the eggs were dispersed evenly in the Petri dishes (Fig.1) while there was minimal contact to facilitate removal of non-viable eggs and reduce the probability of transmission of possible fungi into the dishes. This precautionary action may have prevented possible mortality of developing embryos. Egg-to-egg contact can promote the rapid spread of infectious microorganisms and the presence of non-viable eggs in the incubation conditions could lead to transmission of bacteria to viable and developing embryos.

Before the experiment was initiated, there was assessment to determine the optimal frequency for water replacement for the samples and it was determined that water changes between 24–48 h post-fertilization resulted in a reduction in percentages of developing embryos. Based on recommendations (Pšenička M. and Franěk, R. unpublished data), there was limiting of regular water exchanges until neurulation occurred, after which embryos are less susceptible to disruptions in development after physical manipulation (Dettlaff et al., 1993).

At neurulation of sterlet eggs (about 3 days subsequent to initiation of incubation; 72 h), it is very important to carefully remove the non-viable embryos and to replace the water by flushing the Petri dishes several times. Percentage of embryonic development to the stage of neurulation correlates well with percentage hatching capacity (Dettlaff et al., 1993). As a result of photographic recording, there was facilitation of embryonic development evaluations; initially using an Olympus camera and subsequently with use of mobile phones.

The use of the static tabletop incubation system in an air-conditioned laboratory has two important advantages in comparison to

use of the hatchery tank-incubation method and Q-cell incubator box. Firstly, many different batches of eggs can be examined requiring only controlled-temperature conditions making this incubation technique very economical from a financial perspective. The second major advantage of the static tabletop system is the ease of monitoring and photographing eggs during the period when development is supposed to occur. Furthermore, eggs in Petri dishes do not need to be transferred into a culture chamber for microscopic examination or photographic recording. There, however, is a disadvantage when there is use of the air-conditioned laboratory method because of the initial amount of time required to load the Petri dishes and plastic boxes. For research purposes, the advantage of this process, in terms of ease of frequent monitoring of the same eggs and larvae during a study, more than offsets the initial investment in loading time.

5. Conclusion

In the present study, this is the first time there was evaluation and comparisons of three types of egg incubation techniques. The use of static tabletop Petri dish incubation in an air-conditioned laboratory is an effective means of incubating sterlet eggs with the procedures being simple and generally applicable to laboratory conditions.

CRediT authorship contribution statement

Yu Cheng: Software, Resources, Data curation, Investigation, Validation, Writing - review & editing, Formal analysis. **Miaomiao Xin:** Investigation, Conceptualization, Resources, Supervision, Writing - review & editing. **David Gela:** Resources. **Marek Rodina:** Resources. **Vladimíra Tučková:** Resources, Investigation, Project administration. **Vojtěch Kašpar:** Resources, Writing - review & editing. **Mohammad Abdul Momin Siddique:** Writing - review & editing. **William L. Shelton:** Writing - review & editing, Validation. **Otomar Linhart:** Conceptualization, Investigation, Methodology, Writing - original draft, Project administration, Validation, Supervision, Funding acquisition.

Declaration of Competing Interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2020.106334.

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CHAPTER 3

STANDARDIZATION OF EGG ACTIVATION AND FERTILIZATION IN STERLET (*Acipenser ruthenus* L.)

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My share on this work was about 40%.

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Standardization of egg activation and fertilization in sterlet (*Acipenser ruthenus*)

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ABSTRACT

This study was conducted to determine the minimum number of spermatozoa and optimal volume of activation water for effective fertilization of sterlet *Acipenser ruthenus* eggs. It was also to determine the minimum concentration of spermatozoa per ml of activation water during artificial propagation, when sperm with good motility and velocity (95 % motility and 150 μm s^{-1} curvilinear velocity) were used for fertilization. In three experiments, eggs (~50) from three sterlet were fertilized by pooled sperm from four males in four replicates and incubated at 17 °C. The success of fertilization was evaluated at neurulation and hatching. Sterlet eggs were fertilized in proportion to the concentration of spermatozoa per ml in activation water. A total of 50,000 spermatozoa in 8 ml or 32 ml of activation water (concentrations of 6,250 and 1,563 spermatozoa per ml of activation water, respectively) was sufficient for successful fertilization based on neurulation and hatching rates. Neurulation and hatching gradually decreased with a decrease of the spermatozoa ÷ egg ratio from 6,250 to 114. A ratio 1 ÷ 4 between eggs and water was insufficient to provide adequate fertilization. Highest neurulation and hatching ratios were reported when the egg ÷ water ratio reached 1 ÷ 15, which is recommended for practical usage. Our results therefore showed that a very low concentration of 100 – 1,300 spermatozoa per ml with around 95 % motility and curvilinear velocity over 150 μm s^{-1} in activation water can provide effective fertilization. In studies of fishes with external fertilization, the volume of activation water during fertilization, total number of spermatozoa and eggs and/or concentration of sperm in activation water should be defined.

1. Introduction

Sturgeons (Chondrostei, Acipenseridae) are ancient fish species widely known for their caviar. Most sturgeon species have been negatively affected by anthropogenic activities, including overexploitation; many are critically endangered. Artificial propagation of sturgeons is well established and there is extensive knowledge of all aspects of their reproductive biology This is relevant because of the decline of natural populations and the increasing importance of restocking and other management programs. The sterlet *Acipenser ruthenus* L., 1758 is a common Eurasian sturgeon with a relatively small body size and consequently one of the shortest reproductive cycles among the group (Dettlaff et al., 1993). Artificial reproduction of sterlet is well established and availability of captive populations makes this species ideal as a model for Acipenseridae in general.

Compared to other freshwater bony fishes, sturgeon spermatozoa exhibit several specific differences (e.g. presence of an acrosome) and are motile for several minutes (Linhart et al., 1995b; Tsvetkova et al., 1996; Cosson et al., 2000). In contrast to the eggs of teleosts, sturgeon eggs have several micropylar structures (4–36) and these remain permeable for a few minutes after laying; thus relatively fewer sperm cells are needed for fertilization (Dettlaff et al., 1993; Alavi et al., 2019). Consequently, the strategy of reproduction and gamete interaction may differ from other freshwater fish species. Sturgeon eggs are considerably more resistant to the effects of water exposure than eggs of cyprinids or salmonids (Ginsburg, 1972). The eggs of the latter are affected immediately upon contact with water and lose their fertilization ability within 30 – 60 s. Basic procedures for artificial propagation of sturgeons have been described (Dettlaff et al., 1993; Chebanov and Galich, 2011), including the recommendation to dilute sperm with

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water prior to fertilization (adding to batch of eggs) so as to ensure good fertilization but avoiding polyspermy (Ginsburg, 1972; Iegorova et al., 2018). Another difference in the propagation protocols for salmonids and cyprinids is the volume of water used for activation of gametes and creating suitable conditions for fertilization. For freshwater bony fishes, a volume of water lower than the volume of eggs is usually added (Linhart et al., 1995a; Coward et al., 2002), while for sturgeons, water volume four times higher than the volume of eggs is used (Chebanov and Galich, 2011).

The control of reproduction is critical in aquaculture. The processes of egg activation and fertilization are highly important issues in fish reproductive biology. One of the limiting factors of reproductive success is the quality of gametes (Rurangwa et al., 1998; Coward et al., 2002; Alavi et al., 2008; Bobe and Labbé, 2010). Fish sperm quality is highly variable among different males and from one collection to the next in the same individual (Alavi et al., 2008, 2019). It is becoming increasingly apparent that sperm quality plays a greater role in determining fertilization success than previously thought (Snook, 2005). The question is: "Which parameters should we investigate during *in vitro* fertilization?" Fertilization conditions are usually described in relation to the optimum spermatozoa ÷ egg ratio (Butts et al., 2009, 2014; Gallego et al., 2013; Nynca et al., 2014, 2015; Blawut et al., 2018; Kristan et al., 2018; Beirao et al., 2019; Ramos-Judez et al., 2019). Beirao et al. (2019) reported that an optimum fertilization environment differs among various economically important fish species, from a very low spermatozoa ÷ egg ratio of 10^2-10^3 spermatozoa per egg in European catfish *Silurus glanis* L., 1758 (Linhart et al., 2004) or African catfishes *Clarias* sp. (Rurangwa et al., 1998) to high spermatozoa ÷ egg ratios as in cyprinids where 10^5 spermatozoa per egg is used in species such as barb *Barbus barus* L., 1758) or roach *Rutilus meidingerii* (Heckel, 1851) (Lahnsteiner et al., 2003). A more interesting parameter is the concentration of spermatozoa in the water during fertilization, as stated by Ginsburg (1972) in lake trout *Salvelinus namaycush* (Walbaum, 1792) and Russian sturgeon *Acipenser gueldenstaedtii* (Brandt & Ratzeburg 1833). Ginsburg (1972) states that for good fertilization in sturgeon, a sperm concentration of 10^5 per ml in activating water is sufficient. In contrast, for lake trout, concentrations of 10^7 - 10^9 per ml of activating water is recommended. In carp *Cyprinus carpio* L., 1758, Linhart et al. (2015) found that good fertilization of eggs is achieved with 2×10^4-10^5 spermatozoa per egg, in water for activation of gametes at a concentration 1.6×10^7 spermatozoa per ml, which is not very different from the values reported by Ginsburg (1972) for lake trout. The total number of spermatozoa which Linhart et al. (2015) used for fertilization, however, was huge at 8×10^7 spermatozoa with 5 ml of activation water. Usually, these total quantitative values of sperm are not reported although these data are very important for all studies dealing with sperm quality (Siddique et al., 2015; Alavi et al., 2019; Beirao et al., 2019). The effect of sperm count on fertilization capability in sturgeons has not yet been studied in detail.

The aim of this study on sterlet fertilization was to determine the minimum number of spermatozoa with optimal volume of activation water, *i.e.* the minimum concentration of spermatozoa per ml of activation water, using sperm with good motility and velocity.

2. Materials and methods

Experiments with fish were specifically approved by the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany, based on the EU-harmonized Animal Welfare Act of the Czech Republic.

2.1. Broodstock handling and gametes collection

Sterlet males weighing 0.8–1.9 kg and females weighing 1.5 − 2 kg were obtained from the experimental station of the Faculty of Fisheries and Protection of Waters, the University of South Bohemia at Vodnany, Czech Republic. Prior to experimentation, male and female broodstock were kept in separate 4 m^3 hatchery tanks with a water flow rate of 0.2 L s^{-1}, O_2 of 7.0 mg L^{-1} and water temperature of 15 °C.

Eighteen females (six per experiment) were used. Ovulation was induced with carp pituitary extract dissolved in 0.9 % (w/v) NaCl solution; 0.5 mg kg^{-1} body weight was injected as a priming dose and a second injection of 4.5 mg kg^{-1} body weight was given 12 h later. Eggs were collected 30 h after the second injection. Ova were stripped gently following standard procedures and were kept in plastic bowls. Eggs from three individual females per experiment were visually chosen as the best and used individually within 1 h of collection for each fertilization experiment assay. The criteria for selection of eggs was those with a darker coloration (*i.e.* the darker the more desirable) and when there was no decay.

A single intramuscular injection of carp pituitary extract was administered to each of 24 males (eight males for each experiment) at 4 mg kg^{-1} body weight and a plastic catheter (4 mm diameter) was used to collect milt 48 h after injection, then stored at 4 °C. The sperm motility was checked by microscope and milt from four males per experiment with subjectively assessed good sperm motility (> 95 %) was pooled in equal proportions. Prior to fertilization, the concentration of pooled sperm was determined by a Burker cell hemocytometer (Marienfeld, Germany) (12 squares counted for each male) under an Olympus microscope BX 41 (4009) and expressed in billions per milliliter.

2.2. Spermatozoa motility and velocity assessment

Ten mM Tris–HCl buffer, pH 8 with 0.25 % Pluronic F-127 (prevents sperm from sticking to the slide) was used as the activating medium. The motility of pooled spermatozoa from four males was recorded microscopically (UB 200i, PROISER, Spain) at 10× using a phase-contrast condenser and an ISAS digital camera (PROISER, Spain) set at 25 frames s^{-1}. Recordings were stored on a hard disk in AVI format. Analyses of the sperm recordings were performed by the Integrated System for Semen Analysis software (PROISER, Spain) at 15 s post-activation. Computer-assisted sperm analysis (CASA) included percentage of motile sperm (%) and curvilinear velocity (VCL, μm s^{-1}) were determined. Analyses were carried out in triplicate. All observations were performed at room temperature.

2.3. Fertilization of eggs

Eggs (0.6 g, ~50 eggs; a mean ± SD volume of 0.55 ± 0.06 ml) were inseminated with a corresponding volume of sperm (see description of experiments in 2.5 − 2.7) by pipetting for the fertilization assay in a 25 mL beaker that was placed on a shaking table. Sperm was not added directly to the eggs, but the pipette was inserted to the bottom of the jar next to the eggs. This procedure was repeated four times, therefore, finally, there were four beakers on the shaking table at 17 °C. Then, the mixing table was turned on for 2 min (150 rpm) and mL of water (see description of experiments in 2.5 − 2.7) was pipetted into each beaker. Then, the fertilized eggs were gently distributed in four glass Petri dishes (each beaker was replaced with a Petri dish). Each was filled with dechlorinated water. The 95 mL Petri dishes were 9 cm in diameter and 1.5 cm in depth.

The activation time of 2 min used in fertilization does not reflect the time of spermatozoa motility. Only a small percentage of the spermatozoa remained motile after 3 − 4 min with very low velocity. Shorter fertilization time than motility duration time of spermatozoa was used because with large volumes of activation water, the eggs began sticking to each other after 2 min and therefore the reduction to 2 min. This procedure did not limit fertilization time but because the entire volume of eggs and activation water was poured into the Petri dish, fertilization could have continued without restriction. We assume it was minimal.

2.4. Incubation, neurulation and hatching

Each group of four Petri dishes was kept in four small plastic boxes (13.5 cm × 10 cm × 6.5 cm) one third filled with dechlorinated water (300 mL) which in turn were placed in one larger plastic box (28 cm × 21.5 cm × 7.5 cm; Cheng et al., 2020). The air temperature was maintained at 17 °C. Dechlorinated water was gently changed in the small plastic boxes at 24 h post-fertilization and non-developing embryos were removed; water was exchanged daily after neurulation (72 h post fertilization) up to hatching. The embryo development was recorded using a digital camera (Olympus Corp., Tokyo, Japan, model E-M5MarkII) at 5 h (4 cells) to document the initial total number of eggs, and at 72 h post-fertilization (neurula stage); viable eggs at neurula stages were calculated relative to the initial total number of eggs using our photographic records. Incubation was continued through hatching at 144 h post-activation in experiments 2 and 3. Hatched larvae and malformed larvae were manually counted directly after hatching and compared with the initial number of eggs per Petri dish.

2.5. First experiment

A pool of sperm with sperm concentration of 0.95 × 10^9 was centrifuged at 13,000 × ***g*** for 10 min at 4 °C and the seminal fluid supernatant was obtained. Then, a similar pool of sperm was diluted with seminal fluid 1 ÷ 8 (50 µL sperm + 400 µL seminal fluid). Then the number of 100,000 (0.92 µL), 500,000 (4.59 µl) diluted spermatozoa and 2,500,000 (2.55 µL) with 12,500,000 (12.76 µL) non-diluted spermatozoa in combination with 2, 4, 8 and 16 mL of activation water were applied for fertilization on a pool of eggs from three females, respectively.

2.6. Second experiment

A pool of sperm with a sperm concentration of 0.45 × 10^9 was centrifuged at 13,000 ***g*** for 10 min at 4 °C and seminal fluid was obtained. Later, a similar pool of sperm was diluted with seminal fluid 1 ÷ 9 (100 µL sperm + 900 µL seminal fluid). Then the number of 50,000 (1.11 µL), 250,000 (5.56 µL) and 1,250,000 (27.78 µL) diluted spermatozoa in combination with 2, 8 and 32 mL of activation water were applied for fertilization of eggs from three individual females, respectively.

2.7. Third experiment

At the start a pool of sperm with a concentration 0.49 × 10^9 was centrifuged at 13,000 ***g*** for 10 min at 4 °C and seminal fluid was obtained. A further pool of similar sperm was diluted with seminal fluid 1 ÷ 99 (10 µL sperm + 990 µL seminal fluid). Then the number of 10,000 (2.04 µL) and 50,000 (10.2 µL) diluted spermatozoa with 8 and 88 mL of activation water were used for fertilization of eggs from three individual females, respectively.

2.8. Data evaluation and statistical analysis

Neurulation and hatching rates were evaluated as the effect of total number of spermatozoa in activation water with the volume of activation water (Figs. 1A, 2 A,B, 3 A,B, 4 A, 5 A,B) and also as the number of spermatozoa per mL of activation water (Figs. 1B, 2 C, 3 C,D, 4 B, 5 C,D). In addition, the results in experiments 2 and 3 were evaluated by including all females as repetitions (Figs. 2A,B,C, 4 A,B) as well as in detail in the individual females (the pool of eggs was used only in Fig. 1) (Figs. 3A,B,C,D, 5 A,B,C,D).

Statistical significance was assessed using one-way and multiple ANOVA (SPSS 15.0 for Windows, see legends in Figs), followed by multiple comparison Tukey range tests. Normality of residuals was assessed with the Shapiro–Wilk test. Mean ± S.D. groups with a common lower cases letter do not differ significantly ($P > 0.05$).

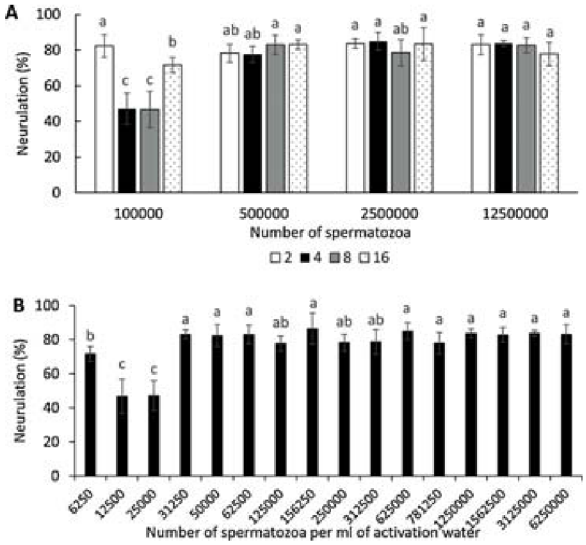

Fig. 1. First experiment showing the neurulation rate (%) of embryos from a pool of eggs of three females using (A) a total number of 100,000, 500,000, 2,500,000 and 12,500,000 spermatozoa in combination with 2, 4, 8 and 16 mL of activation water, and (B) total number of spermatozoa and volume of activation water re-calculated as concentration of sperm per ml of activation water. Mean ± S.D. values of four replicates are shown and are compared using multiple (A) and one-way (B) ANOVA (SPSS 15.0 for Windows), followed by Tukey range tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).

3. Results

Analysis of good quality semen in all three experiments reported 95–99 % motility and 150 − 207 µm s^{-1} VCL at 15 s post-activation. In all three experiments, semen was similar in its percentage of motile spermatozoa but differed in velocity. A higher VCL of spermatozoa was found in the third experiment (207 ± 42 µm s^{-1}) compared to the first (150 ± 39 µm s^{-1}) and second (156 ± 33 µm s^{-1}) experiments.

3.1. First experiment

The results of fertilization of pooled eggs (Fig. 1A) showed that 100,000 spermatozoa in 2 mL of activation water (concentration 50,000 spermatozoa per mL of activation water, Fig. 1B) was sufficient for successful fertilization with 82 % neurulation. On the other hand, a dose 100,000 spermatozoa in increasing volumes of activation water showed a rapid decrease in neurulation to 50 % (4 and 8 mL activation water) and again increased to 85 % neurulation (16 mL activation water). However, from 500,000 to 12,500,000 spermatozoa, the results were stable with 78–85 % neurulation. The effect of sperm concentration per mL of activation water, however, was more consistent (Fig. 1B), but there was also an unexplained fluctuation in low sperm concentration. Stable results in neurulation were achieved only from 31,250 sperm (Fig. 1B). It was found that the number of spermatozoa, the volume of the activation water and sperm concentration on 1 mL of activation water had a significant effect on the neurulation level of embryos.

3.2. Second experiment

For all three females successful fertilization with 69–76 % neurulation and 60–64 % hatching (Fig. 2A,B) resulted when a total of

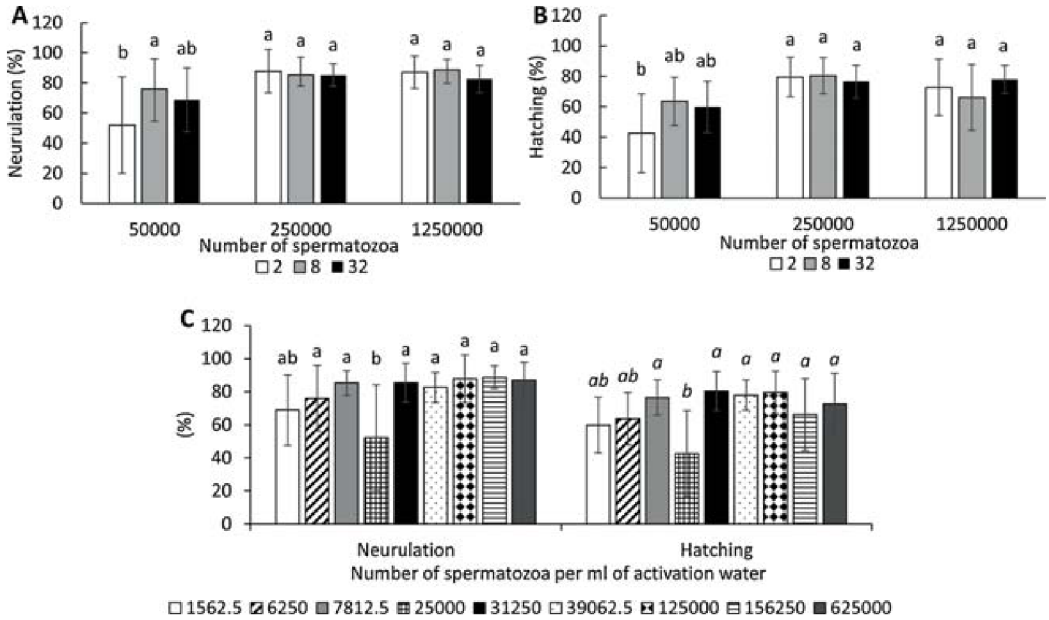

Fig. 2. Second experiment showing (A) (C) neurulation and (B) (C) hatching rates (%) of embryos from eggs of three individual females using (A) (B) a total number of 50,000, 125,000, 625,000 spermatozoa in combination with 2, 8 and 32 mL of activation water and (C) total number of spermatozoa and volume of activation water re-calculated as concentration of sperm per mL of activation water. Mean ± S.D. values with 12 replicates (3 females each 4 replicates) are shown and compared using multiple ANOVA (SPSS 15.0 for Windows), followed by Tukey range tests. Groups with a common lower cases letter do not differ significantly within neurulation and hatching rates ($P > 0.05$).

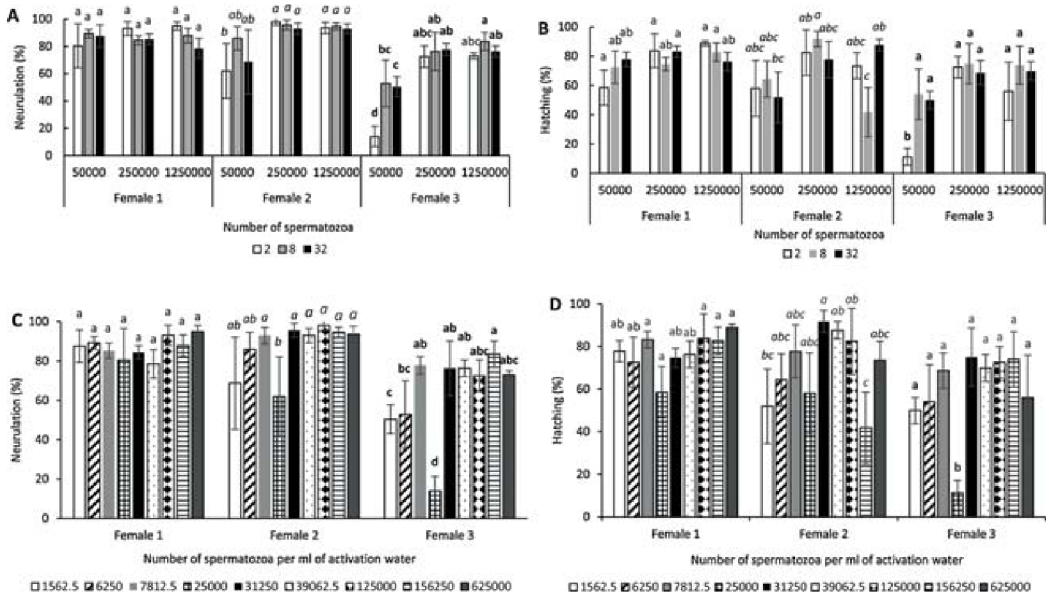

Fig. 3. Second experiment showing (A) (C) neurulation and (B) (D) hatching rates (%) of embryos from eggs of three individual females with (A) (B) a total number of 50,000, 125,000, 625,000 spermatozoa in combination with 2, 8 and 32 mL of activation water and (C) (D) total number of spermatozoa and volume of activation water re-calculated as concentration of sperm per ml of activation water. Mean ± S.D. values are shown and compared using multiple ANOVA (SPSS 15.0 for Windows), followed by Tukey range tests. Groups with a common lower cases letter do not differ significantly within neurulation and hatching rates in each female ($P > 0.05$).

50,000 spermatozoa in 8 mL and 32 mL of activation water (concentration 6,250 and 1,563 spermatozoa per mL of activation water, Fig. 2C) was used. However, 50,000 spermatozoa in 2 mL of activation water had the effect of reducing neurulation and hatching (Fig. 2A,B) and 25,000 spermatozoa per mL of the activation water was the lowest (Fig. 2C). Using 8 and/or 32 mL of activation medium with a total number of spermatozoa from 50,000 to 1,250,000 (concentration 6,250 and 1,563 spermatozoa per mL of activation water) did not affect the level of neurulation and hatching (Fig. 3A,B,C); therefore 8 mL of activation water with the total number of 50,000 spermatozoa

(concentration 6,250 spermatozoa per mL of activation water) was used as a control in the third experiment. It was found that the total number of spermatozoa and concentration of spermatozoa per mL of activation water had a significant effect on the neurulation and hatching level of embryos from all three females. The volume of activation water, however, did not affect neurulation and hatching.

Interestingly, in individual testing of females, no significant effect was observed for the volume of activation medium and quantity of spermatozoa on neurulation and hatching in eggs from female-1 (Fig. 3A,B); this suggested that a minimum of 50,000 spermatozoa in maximal activation volume of water 32 mL (concentration 1,563 spermatozoa per mL of activation water, Fig. 3C,D) was sufficient for egg fertilization and hatching. In theory, this means that a lower number of spermatozoa and higher volumes of activation water would reduce neurulation and hatching in this female. Stable results were also obtained in neurulation in female 2. Unexpectedly using only 2 mL of activation water with a concentration of 25,000 spermatozoa per mL of activation water (Fig. 3C,D) were poorer results observed. Sperm concentration at the level of 1,562 spermatozoa per mL of activation water was sufficient for stable neurulation (Fig. 3C), but in the case of hatching, it was up to 6,250 sperm per mL of activation water (Fig. 3D). In all three females, a high volume of 32 mL activation water with a concentration of 7,812 spermatozoa per mL activation water provided high neurulation and hatching (Fig. 3C,D). In all cases, of a female effect on neurulation and hatching was demonstrated (Fig. 3A,B,C,D).

3.3. Third experiment

Eggs in this experiment were of poorer quality; maximum neurulation and hatching were only achieved at 60 and 56 % of the control, meaning that 50,000 spermatozoa with 8 mL of activation water (Fig. 5A,B), was comparable to a similar sperm count and activation water volume with 89 and 72 % neurulation and hatching (Fig. 3A,B) in the second experiment. On the other hand, the quality of sperm in the third experiment was slightly higher, showing significantly faster velocity than in the second experiment. The results from all three females (Fig. 4A) showed that 10,000 spermatozoa in 8 and 88 mL of activation water (concentration 1,250 and 114 spermatozoa per mL of activation water, Fig. 4B) was sufficient for fertilization with 10–27 % neurulation and 8–22 % hatching. At sperm concentration per mL of activation water, the level of neurulation gradually decreased by using 6,250 to 568 − 1,250 spermatozoa down to the lowest level of 114 spermatozoa (Fig. 4B). It was found that the total number of spermatozoa, the volume of the activation solution, and the concentration of spermatozoa per mL of activation water had a significant effect on the neurulation and hatching levels from all three females.

Almost all individual values in females-1 and -2 (Fig. 5A,B,C,D) showed a clear difference in the level of neurulation and hatching using different total number of spermatozoa, activation water volumes, or sperm concentration per mL of activation water. In female 3, neurulation and hatching values were very low and indicated poor egg quality. Again, the effect of females on achieving neurulation and hatching was demonstrated.

4. Discussion

During the experiment, only semen with a high percentage of motile spermatozoa (above 95 % according to CASA evaluation) and good parameters of motility in terms of velocity (average velocity from 150 to 207 μm s^{-1} at 15 s post spermatozoa activation) was used. The maximum velocity was measured up to 240 μm s^{-1}. Similar values are usually measured for high quality semen of sturgeons immediately after sperm activation (Linhart et al., 1995b; Cosson et al., 2000; Boryshpolets et al., 2013). The spermatozoa of sterlet still exceed a velocity of 120 μm s^{-1} 1 min post-activation (Boryshpolets et al., 2013). Considering that the multiple micropylar holes in the egg

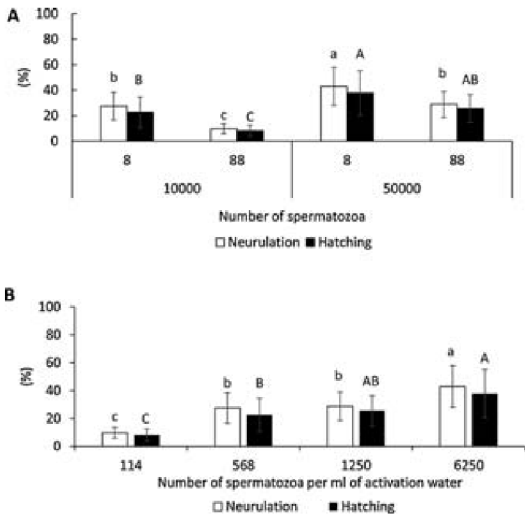

Fig. 4. Third experiment showing combined results of neurulation and hatching rates (%) of embryos of eggs from three females with (A) a total number of 10,000 and 50,000 spermatozoa in combination with 8 and 88 mL of activation water and (B) total number of spermatozoa and volume of activation water recalculated as concentration of sperm per mL of activation water. Mean ± S.D. with 12 replicates (3 females each 4 replicates) are shown and compared using multiple ANOVA) (SPSS 15.0 for Windows), followed by Tukey range tests. Groups with a common lower cases letter do not differ significantly within neurulation and hatching rates ($P > 0.05$).

provide a larger area for sperm entry and the long duration of high velocity and motility of acrosome-equipped spermatozoa, these gametes present a conveniently complex environment to increase the probability of fertilization, even with a very low spermatozoa count.

It has been shown that thousands and even hundreds of spermatozoa in activation water are capable of fertilizing sterlet eggs proportionally according to their concentration in activation water, if the quality of spermatozoa is superior in terms of motility and velocity. The level of fertilization was different depending on the females. Fertilization and hatching tests were conducted within the experiment to characterize the quality of the gametes. A very important factor is if eggs from different females are pooled or if eggs of individual females are fertilized separately. Obviously, in the first case, a large number of samples can be tested, but in our case, the resolution of sterlet spermatozoa counts was very heterogeneous especially at the lowest total number of spermatozoa number (100,000) or in concentration at activation water from 6,250 to 25,000 spermatozoa per mL (Fig. 1A,B). Fertilization was not as much affected by sperm quantity, but rather by poor homogeneity in pooled eggs (Cheng et al., 2020). In the case of numbers higher than 31,250 spermatozoa per mL of activation water (Fig. 1B), such a variability of fertilization success was not observed. This means that in lower concentrations than 31,250 spermatozoa per mL of activation water, there was probably a sperm-egg incompatibility (Snook et al., 2009; Zadmajid et al., 2019), where only certain sperm in a pooled sample of four males were compatible with certain eggs. Reducing suitable spermatozoa for fertilization below the limiting level, in combination with the non-homogeneous representation of eggs from different females, yielded curious results, with high fertilization in the lowest and highest volumes of activation water and very low fertilization in volumes between them.

In Experiment 2 (Fig. 3A,B) with 2 mL of activation water when a total spermatozoa count of 50,000 was used, a reduction in neurulation and hatching was always achieved in 2 females: females 2 and 3. If higher volumes of 8 mL and 32 mL were used with the same number of spermatozoa, the lower concentration of spermatozoa per mL of

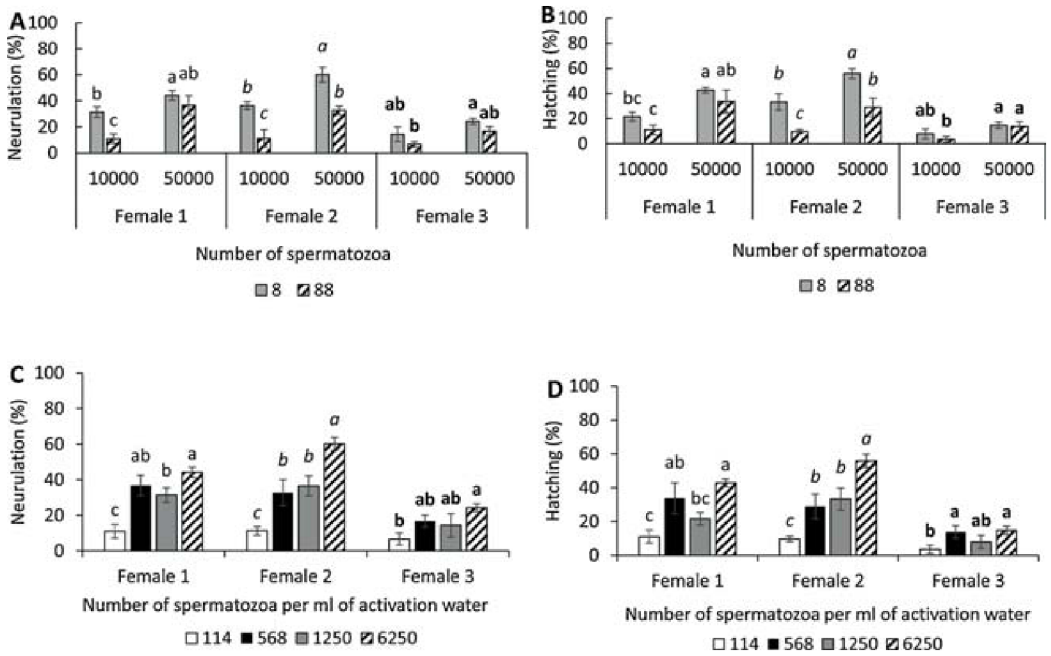

Fig. 5. Third experiment of (A) (C) neurulation and (B) (D) hatching rates (%) from embryos of eggs of three individual females with (A) (B) a total number of 10,000 and 50,000 spermatozoa in combination with 8 and 88 mL of activation water and (C) (D) total number of spermatozoa and volume of activation water re-calculated as concentration of sperm per ml of activation water. Mean ± S.D. values with four replicates are shown and compared using multiple ANOVA (SPSS 15.0 for Windows), followed by Tukey range tests. Groups with a common lower cases letter do not differ significantly within neurulation and hatching rates in each female ($P > 0.05$).

activation water was always better. On the other hand, using a larger quantity of sperm (250,000 or 1,250,000 total number of spermatozoa), this paradox did not manifest itself. This tendency was shown repeatedly, which indicates that it was not an effect of gamete handling or inconsistency of experimental design. We suggest that an insufficient dilution of ovarian fluid by activation water could hinder full spermatozoa activity. This was noticeable when using a low sperm count, so as not to be masked by a high sperm count. Ovarian fluid of sterlet has an osmolality of 190 mOsmol kg^{-1} with a high potassium concentration of 6 mmol L^{-1} (Siddique et al., 2016). A concentration of > 0.5 mmol L^{-1} potassium prevents motility of white sturgeon *Acipenser fulvescens* (Rafinesque, 1817) spermatozoa and paddlefish *Polyodon spathula* (Walbaum, 1792) (Toth et al., 1997; Linhart et al., 2002). Generally, a mass of eggs stripped from sterlet females contains 10–30 % of ovarian fluid, depending on the female, timing of reproduction and also seasonality. In our case, the dilution of the eggs (0.6 g, meaning 0.55 ml) with water (2 mL) was at the activation rate of 1 ÷ 3.64 and ovarian fluid made up 3–10 % of the total volume. In this case, the potassium level could be 0.5 mmol L^{-1}, which could significantly affect sperm motility in sturgeons (Toth et al., 1997; Linhart et al., 2002). It was probably the higher content of ovarian fluid in eggs of females 2 and 3 (Fig. 3A,B) that caused lower neurulation and hatching. When such a rate of 1 ÷ 3.64 was compared to the usual dilution in practice of 1 part eggs + 4 parts water (Dettlaff et al., 1993), the ratio in the experiment was not much different. The fertilization was most successful when 0.6 g (0.55 ml) of eggs were fertilized using 8 mL of activation water, which means a ratio of 1 ÷ 14.54 (1 ÷ 15), which we recommend for practical purposes. The ratio of 1 ÷ 4 commonly used for artificial reproduction is too low and does not ensure the full potential for fertilization or consistency of fertilization results with batches of eggs containing a large proportion of ovarian fluid.

In all cases, the usual dilution of sperm during egg fertilization which is used for elimination of polyspermy is 1 ÷ 100 − 200 (Dettlaff et al., 1993; Iegorova et al., 2018). In our conditions it was a ratio from 1 ÷ 157 to 1 ÷ 4,400,000. It is not likely that large volumes of water under natural spawning conditions, somehow reduces the success of fertilization, which is a relatively rare finding in fishes. On the contrary, it seems that a large dilution of semen and eggs is suitable for fertilization in sturgeons.

An optimum spermatozoa ÷ egg ratio is believed to be a crucial factor for standardization of *in vitro* fertilization in research studies as well as practical reproduction conducted at fish farms (Butts et al., 2009, 2014; Gallego et al., 2013; Nynca et al., 2014, 2015; Blawut et al., 2018; Beirao et al., 2019; Ramos-Judez et al., 2019). However, this kind of description of the fertilization environment is not complete and there is a possibility of misleading application if no other data are known or reported, *e.g.* volume of activation water and number of eggs used for fertilization. Therefore, a combination of all parameters is important for fertilization, and rough calculations of spermatozoa ÷ egg ratio are not sufficient to accurately describe actual fertilization conditions. Moreover, it is very important that the sperm quality is determined in detail depending on the different gamete parameters (Beirao et al., 2019). We must not forget that the spermatozoa ÷ egg ratio parameter originated from studies in mammals, especially from cattle breeding programs in Denmark in 1936 (Foote, 2002); here it was defined as to how many spermatozoa must be in one insemination dose to fertilize a female. In the case of internal insemination, the internal parameters in the female are clearly defined as species specific (internal volume, composition, *etc.*), as opposed to external fertilization in fishes, where fertilization must always be defined precisely in the study, meaning the volume of water during fertilization, the number of sperm and eggs and/or concentration of sperm per mL of activation water. The concentration of sperm and eggs in water during fertilization plays a crucial role in fertilization. It is astonishing that this parameter is not mentioned in the literature and we are generally satisfied only with the sperm count parameter per egg, omitting the calculations in which the

gametes are found in fertilization. Using the spermatozoa ÷ egg ratio as an exclusive parameter to describe the fertilization environment in fishes is inappropriate. Using carp as an example, Linhart et al. (2015) determined that for effective fertilization it is sufficient to use 2×10^4-10^5 spermatozoa per egg, but in fact, the total number of spermatozoa used for fertilization was huge, 8×10^7 spermatozoa. A low spermatozoa ÷ egg ratio in fertilization trials was achieved due to the use of a high number of eggs of 4000 and a minimum volume of activation water. If ten times the number of eggs were used, it would probably produce similar results but with a lower spermatozoa ÷ egg ratio. A similar result could also be expected in the above-mentioned sterlet experiments when a ratio of 1 ÷ 1000 (egg ÷ spermatozoa) was achieved using 50,000 spermatozoa and 50 eggs. In fertilization, however, not 0.6 g (~50) eggs but 6 g (~500) eggs could be used and then the ratio would be 1 ÷ 10,000. We believe that it is important to clearly determine the volume of activation water, the number of spermatozoa and eggs to reach good level of fertilization. Using such parameters, we approach the normal natural reproductive conditions for which both sexes have adapted through evolution (Ginsburg, 1972) and therefore, each species significantly differs in fertility and has different times for which it is possible to fertilize eggs. When the female is spawning with males naturally, both gametes are dispersed at a certain concentration, which may be crucial for good fertilization and development. On the other hand, studies of post-copulatory sexual selection reveal that sperm numbers are not the exclusive predictor of competitive fertilization success. Males have an extensive arsenal of sperm traits that they use in paternity contests and females influence the outcome of sperm competition (Snook, 2005).

In the mammalian literature, methodologies for insemination/fertilization always contain data on the number of spermatozoa used, the so-called insemination dose (Foote, 2002). In fishes such information is in the older literature and is usually part of manuals/methodologies for reproduction of species, where mostly only the volume of sperm is stated (Billard, 1999; Mims and Shelton, 2015). This is insufficient without data on sperm concentration in mL of semen. Therefore, we recommend that the total number of spermatozoa or, similarly to Ginsburg (1972), the concentration of spermatozoa per mL of activation water should be given to explain fertilization. Ginsburg (1972) states that a sperm concentration of 10^5 per mL of activation water is sufficient for good fertilization in sturgeons. In our case it was only 10^3 spermatozoa per mL which were satisfactory for good fertility.

The quality of semen in sturgeons in connection with fertility has been unexplored. Sperm quality as defined by Bobe and Labbé (2010) is its ability to successfully fertilize an egg and subsequently allow the development of a normal embryo. In aquaculture the quality of fish gametes can be highly variable and in broodstock management practices is under the influence of a significant number of external factors. For this reason, the gamete quality has received increasing attention (Bobe and Labbé, 2010). However, no attention has been paid to the difference in fertility of sperm with excellent or poor motility and velocity and what quantity of sperm is needed at low sperm quality. This information would be very important for farmers if they knew how much semen to use with reduced motility. For the time being, only intuitive values have been used, with a higher volume/quantity of semen of inferior quality being used. In sturgeons, due to the high fertility of spermatozoa, this indicator seems to be even more negligible from a practical point of view. However, we know from practice that the quality of sterlet semen is quite variable and usually in 10 males, only semen of one to four males exhibits motility above 90 % and the remainder have motility from 0 to 60 % (Xin et al., unpublished). Paradoxically, this high fertility of spermatozoa allows the use of spermatozoa with poor motility, for example at the level of 10–30 % motility, but using an increased number of spermatozoa. This needs to be defined in the future.

5. Conclusion

It has been successfully demonstrated that thousands and even hundreds of spermatozoa with a minimum 95 % motility and 150 μm s^{-1} velocity are able to fertilize sterlet eggs proportionally, according to their concentration in activation water. In order to achieve adequate fertilization, it is always necessary to use eggs from individual females in sturgeons. A total of 50,000 spermatozoa in 8 mL or 32 mL of activation water (concentration 6,250 and 1,563 spermatozoa per mL of activation water) was enough for successful neurulation and hatching. The neurulation and hatching gradually decreased with a decrease of the spermatozoa ÷ egg ratio from 6,250 to 114. The ratio between eggs and water of 1 ÷ 4 is insufficient for good fertilization and this ratio is most often used for mass reproduction in sturgeons. Neurulation and hatching were most successful when the ratio between eggs and water for activation was 1 ÷ 15. Such a ratio is recommended for fertilization under farming conditions. A large dilution of sperm and eggs is suitable for fertilization in sturgeons.

Declaration of Competing Interest

All authors declare no conflicts of interest. All authors listed in the manuscript contributed to preparing the manuscript and attest to the validity and legitimacy of the data and its interpretation and agree to its submission to your journal.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100381.

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CHAPTER 4

SPERM MANAGEMENT AND FERTILIZATION OF ZEBRAFISH (*Danio rerio* H.), EUROPEAN CATFISH (*Silurus glanis* L.), AND STERLET (*Acipenser ruthenus* L.)

4.1. Cheng, Y., Franěk, R., Rodina, M., Xin, M.M., Cosson, J., Zhang, S.P., Linhart, O., 2021. Optimization of sperm management and fertilization in zebrafish (*Danio rerio* (Hamilton)). Animals 11, 1558.

My share on this work was about 70%.

4.2. Linhart, O., Cheng, Y., Rodina, M., Gela, D., Tučková, V., Shelton, W.L., Tinkir, M., Memiş, D., Xin, M.M., 2020. Sperm management of European catfish (*Silurus glanis* L.) for effective reproduction and genetic conservation. Aquaculture 529, 735620.

My share on this work was about 40%.

4.3. Xin, M.M., Cheng, Y., Rodina, M., Tučková, V., Shelton, W.L., Linhart, O., 2020. Improving motility and fertilization capacity of low-quality sperm of sterlet *Acipenser ruthenus* during storage. Theriogenology 156, 90–96.

My share on this work was about 30%.

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Article

Optimization of Sperm Management and Fertilization in Zebrafish (*Danio rerio* (Hamilton))

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Simple Summary: For scientific studies on the zebrafish model, simple and routine reproductive procedures should be used to ensure stable and repeatable results. When the milt is collected, spermatozoa are spontaneously activated by urine or excrement (low osmolarity) which routinely contaminates the samples, because of the minuscule size of the fish body. Therefore, whenever milt is collected from a zebrafish for short-term milt preservation and artificial fertilization, milt must be collected into an immobilizing solution, which because of its high osmolarity stops the movement of spermatozoa and keeps the sperm immobile until fertilization. Usually, the spermatozoa showed forward movement during the 35 s period following dilution in water. The sperm concentration ranged from 0.08 to 3.52 × 109/mL with a volume from 0.1 to 2.0 µL per male. The most suitable extender proved to be E400, which allowed storage of sperm for fertilization for 6 to 12 h at a temperature of 0–2 °C. To achieve a good level of fertilization and hatchability, a test tube with a precisely defined amount of sperm with extender, eggs and activating solution proved to be the most effective.

Abstract: The aim of the present study was to investigate the spontaneous motility of spermatozoa and to optimize sperm collection, short-term sperm storage, and fertilization in zebrafish *Danio rerio*. The movement of spermatozoon in water was propagated along the flagellum at 16 s after sperm activation then damped from the end of the flagellum for 35 s and fully disappeared at 61 s after activation. For artificial fertilization, milt must be added to an immobilizing solution, which stops the movement of sperm and keeps the sperm motionless until fertilization. E400 and Kurokura as isotonic solutions were shown to be suitable extenders to store sperm for fertilization for 6 h. E400 stored sperm for 12 h at 0–2 °C. Sperm motility decreased only to 36% at 12 h post stripping for the E400 extender and to 19% for the Kurokura extender. To achieve an optimal level of fertilization and swim-up larvae rates, a test tube with a well-defined amount of 6,000,000 spermatozoa in E400 extender per 100 eggs and 100 µL of activation solution has proven to be more successful than using a Petri dish. The highest fertilization and swim-up larvae rates reached 80% and 40–60%, respectively, with milt stored for 1.5 h in the E400 extender at 0–2 °C.

Keywords: zebrafish; *Danio rerio*; sperm motility; fertilization; short-term storage; extender

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1. Introduction

The zebrafish (*Danio rerio* (Hamilton)) is a popular freshwater fish belonging to the minnow family Cyprinidae of the order Cypriniformes, which has been extensively used as an invaluable vertebrate model organism for study in scientific laboratories since the late 1960s. Over the past few decades, laboratories across the world have produced numerous

mutant strains and transgenic and wild-type zebrafish lines. As a model fish, it has the following advantages: easy and cheap maintenance in aquarium recirculation systems, short generation time, full genome map, year-round spawning, and rapid development. In particular, compared to larger fish species such as rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*), it does not need breeding with hormonal induction [1,2]. In view of these advantages, it is appropriate to study gametes using zebrafish as a model organism for subsequent research, for example, into functional genomics [3], physiology [4], environmental monitoring [5], and human diseases [6]. It was found that the DNA methylome of sperm, which is crucial for embryonic development [7,8], was inherited in early zebrafish embryos. However, gamete handling in zebrafish as a model organism needs to be improved, as it provides an important basis for further research into epigenetics, in vitro fertilization, cryopreservation, artificial polyploidy, and uniparental inheritance induction.

It is known that the spermatozoa of most freshwater fishes are immotile in the testes and seminal plasma due to the level of osmolality and the composition of seminal plasma [9,10]. Activation occurs when spermatozoa are released into a hypotonic aqueous environment during spawning [9,10]. For zebrafish, sperm motility is initiated in hypotonic solutions at a wide range of osmolalities (0–270 mOsm/kg) [11–13] with the highest sperm motility at 150–210 mOsm/kg [13]. A few studies in freshwater species have shown that sperm can be contaminated with urine during collection by stripping. This results in spontaneous movement of spermatozoa in the collected milt of some cyprinid species such as common carp [14], tench (*Tinca tinca*) [15], asp (*Aspius aspius*) [16], and zebrafish [17,18]. In the case of large species of fishes, excrement or urine can be separated from the milt during collection [19]. In small fishes, these components cannot be separated when stripping through the genital papilla and must be collected (see Video S1). Therefore, a higher incidence of milt contamination is possible in these small-bodied species. To prevent spermatozoa spontaneous movement due to low osmolarity of the urine or excrement, which results in a rapid decrease in fertility, it is important to collect milt in a suitable extender (immobilizing solution), which inhibits sperm movement and preserves its fertilizing ability [17,18,20,21]. After milt collection, the zebrafish laboratory guides recommend time limits to use gametes efficiently. These aging limits are 1.5 or 2 h for sperm stored in Hanks' buffered salt solution on ice [22,23] and 2 h for eggs stored in a Hanks' modified medium at room temperature [24]. Cardona-Costa et al. [25] indicated that the temporal limits usually recommended for zebrafish milt to fertilize fresh eggs could be extended for up to 24 h without significant differences, compared with fresh sperm in a modified medium (Hanks' saline solution supplemented with 1.5 g BSA and 0.1 g ClNa, pH 7.4). There are several previously published extenders that show good short-time milt storage (from 6 to 24 h) such as Hanks' balanced salt solution (HBSS) [26], modified Hank's medium (MHBSS) [25], and the E400 extender [18].

Therefore, our goal was (1) to verify whether the phenomenon of spontaneous motility exists in zebrafish in general or only rarely, (2) to adjust the method of milt collection, (3) to test which extender is most suitable for milt management by the sperm motility parameters, fertilizing ability, and swim-up larvae rates, and (4) to optimize the fertilization methods.

2. Materials and Methods

2.1. Ethics Statement and Animals

The study was conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Vodňany, Czech Republic. The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016-17214). The methodological protocol of the current study was approved by the expert committee of the Institutional Animal Care and Use Committee of the FFPW according to the law on the protection of animals against cruelty (reference number: MSMT-6406/119/2).

2.2. Fish Culture and Stripping

Zebrafish males (8–12 months old) were used and cultured in eight l aquaria with females separate. The male AB reference strain was used (wild type) from the European Zebrafish Resource Center, and the transgenic line vasa EGFP (basis from AB strain), expressing enhanced green fluorescent (EGFP) protein in germ cells, was exclusively purchased from the University of Liège, Belgium. An approximate sex ratio was 1:1, at a density of three fish per l at 28.5 °C in a recirculating water system. The photoperiod was set at 14 h light: 10 h dark, and fish were fed twice per day with Gemma micro 500 (Skretting, Norway) and once per day with baby brine shrimps (*Artemia* spp.).

Before milt collection, males were housed with females overnight in spawning aquaria as described in Franěk et al. [2]. In total, a set of 300 males and 10 females were anesthetized during these experiments, and among those, milt was collected from 200 males; the rest of the males ejaculated an insufficient volume of milt and were not included in the analysis. Males and females were anesthetized in TRIS buffered 0.05% tricaine methane sulfonate solution (MS-222; Sigma-Aldrich, St. Louis, MO, USA, E10521-10G) and briefly dipped in the aquarium water. The area of the fish's genital papilla was gently dried with tissue paper, the fish was placed on tissue paper in dorsal recumbency (belly up), and milt was collected (Video S2). The eggs were pooled during collection and fertilized promptly (Video S3). Immediately after stripping and collection of gametes, fish were then transferred into fresh water for recovery and were not used any further in this study (Videos S2 and S3).

2.3. Experimental Design

The primary design of the experiment was as follows:

(1) Testing spontaneous movement of spermatozoa.
(2) Basic screening of four extenders from the literature with selections of the two most robust for further studies.
(3) Detailed study of two extenders on how they are able to maintain motility and fertility depending on the storage period. Sperm motility, concentration, and seminal plasma osmolality were assayed using (a) milt from individual males, (b) pooled milt at time of collection, (c) individually collected milt and a storage pool of spermatozoa with good motility. In addition, visualization of the detailed movement of spermatozoa was performed using a high-speed video microscopy and a stroboscopic lamp. In the case of fertility, individually collected milt and a storage pool of spermatozoa with good motility were tested.
(4) Finally, a suitable fertilization technique was specified.

2.4. First Step: Testing Spontaneous Movement of Spermatozoa

Testing whether spermatozoa are motile or not without activation by water is very important. Milt from five AB strain males was collected individually in a 10 µL micropipette using gentle, bilateral abdominal pressure. A drop of milt was immediately spread onto the surface of a glass slide previously positioned on a microscope stage at room temperature (22–23 °C) and observed at 20× magnification using dark-phase microscopy to evaluate whether global sperm movement occurred. Milt was not placed on ice (0–2 °C) during handling.

2.5. Second Step: Testing Extenders

Fifteen males from the AB strain were used to select the best extenders. Due to the spontaneous movement of spermatozoa in milt collected from all five males as observed in the previous test, improvement of the collection method was needed. The urogenital opening was carefully dried with tissue paper and then was rinsed with the extender to be tested. Milt was rapidly collected in a 10 µL micropipette using gentle, bilateral abdominal pressure and immediately added to 20 µL of the extender to be tested. Milt was collected separately from three males in each of 200 µL test tubes, each one containing 20 µL of the extender to assess the capacity of different extenders to preserve sperm motility.

Milt collected individually from three males without extenders was used as a control group. Twelve males were used to test four extenders (prepared in the laboratory by the authors): (1) (Kurokura) (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, and 2.38 mM $NaHCO_3$) [15], (2) Hanks' balanced salt solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH 7.2, 300 mOsmol/kg) [26], (3) Modified medium (MHBSS) (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 4.2 mM $NaHCO_3$) (100 mL of Hanks' saline supplemented 1.5 g BSA and 0.1 g ClNa, 320 mOsmol/kg, pH 7.4) [24,25], and (4) E400 extender (130 mM KCl, 50 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-(+)-Glucose, and 30 mM HEPES-KOH, pH 7.9, 400 mmol/kg) [18]. All extenders and samples of milt mixed with extenders were stored on ice; their spontaneous motility was estimated without activation by distilled water and then also activated in distilled water at 0.5, 6, 12, and 24 h post-stripping (HPS).

2.6. Third Step: Testing of Sperm Storage, Spermatozoa Concentration, Seminal Plasma Osmolality, and Fertilization with Swim-Up Larvae Rates

2.6.1. Storage of Milt from Individual Males

Nine males including three males from the AB strain and six males from the EGFP strain were used for the milt storage experiment in extenders Kurokura and E400, which were selected as the best from the previous test. The urogenital opening of each male was dried with tissue paper and then its milt was collected directly into a 10 µL micropipette. After stripping the nine males individually, the milt from each male was immediately divided evenly into 200 µL tubes, one containing 20 µL of the Kurokura and the other with 20 µL of the E400. Altogether, there were 18 tubes with approximately 0.15–0.75 µL of milt in each tube; the ratio of milt: extender was 1:27–33 (see Video S2). All samples of milt in extenders were stored on ice; the motility and velocity of the spermatozoa were evaluated at 0.5, 6, 12, and 24 HPS.

2.6.2. Storage of Pooled Milt at Time of Collection

First, milt from one group of 11 AB strain males was one by one separated into one 200 µL tube with 80 µL of E400 and one 200 µL tube with 80 µL of Kurokura (altogether two tubes with approximately 5.5 µL of pooled of milt in each tube; the ratio of milt: extender was 1:15). Second, four groups of 5, 6, 11, and 12 AB strain males were similarly one by one evenly separated into four tubes with 39 µL of E400 and four tubes of 39 µL Kurokura, respectively. Altogether there were eight tubes with approximately 1.55–2.3 µL pooled of milt in each tube; the ratio of milt: extender was 1:17–25. All milt added into tubes with extenders was stored on ice, and the motility and velocity were evaluated at 0.5, 12, and 24 HPS.

2.6.3. Individually Collected Milt and Storage Pool of Spermatozoa with Good Motility

Milt from nine individual AB strain males was evenly divided into 20 µL of E400 (9 tubes) and 20 µL of Kurokura (9 tubes). Then only sperm from six males with motility evaluated as good (above 90%) was pooled in two tubes with Kurokura or E400 extenders (c. 1.9 µL of pooled milt; the ratio of milt: extender was 1:63) and stored on ice. The motility and velocity were evaluated at 0.5, 12, and 24 HPS.

2.6.4. Sperm Motility, Velocity, Concentration, and Osmolality of Seminal Plasma

Distilled water supplemented by 0.25% Pluronic F-127 (catalog number P2443, Sigma-Aldrich) used to prevent sperm from sticking to the slide was used as the activating medium (pH 7.0–7.5) and maintained on ice prior to all experiments. Sperm was activated at room temperature (21 °C) by mixing the diluted sperm sample (0.5–2 µL, according to the density of sperm sample) into 20 µL of the activation medium on a glass slide at 0, 6, 12, and 24 h post-stripping. The activated spermatozoa were directly recorded microscopically (UB 200i, PROISER, Madrid, Spain) at 20× using a dark-field condenser and an ISAS

digital camera (PROISER, Spain) set at 25 frames/s. Analyses of the sperm recordings were performed by the Integrated System for Sperm Analysis software (PROISER, Spain) at 15 s post sperm activation, and 25 frames were used to analysis velocity parameters. Computer-assisted sperm analysis (CASA) included the percentage of motile sperm (%), curvilinear velocity (VCL, μm/s), and straight-line velocity (VSL, μm/s). The scale was calibrated. Escala X and Escala Y were both set up to 0.625 μm when using a 20× lens on a dark field microscope. Quantitative analyses of all samples were conducted in triplicate.

Sperm concentration together with the total number of sperm per male was evaluated for individual males in E400 and Kurokura extender solutions. In addition, spermatozoa of pooled sperm samples were counted. The sperm concentration (expressed as 10^9/mL) was determined by a Bürker cell hemocytometer (Marienfeld, Lauda-Königshofen, Germany; 12 squares counted for each male) using an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) under an Olympus microscope BX 41 (4009).

The osmolality of seminal plasma from 20 males was evaluated. A total of 15 μL of sperm was collected and centrifuged at 17,000× *g* at 4 °C for 5 min (Thermo Scientific, Fresco 21). The seminal plasma (supernatant) of 10 μL was collected and diluted with 50 μL (dilution six times) in distilled water. Finally, seminal plasma osmolality was repeatedly measured three times in a Freezing Point OSMOMAT 3000 (Gonotec, Berlin, Germany), and the mean result, multiplied by six, was expressed in mOsmol/kg.

2.6.5. Evaluation of Sperm Motility by Stroboscopic Illumination

Milt was collected from three males and added to IS E400 (prepared in the laboratory by the authors). The following activation solution was composed of 1 part of IS E400 + 1 part 1% BSA + 5 parts of distilled water. For a detailed visualization of swimming spermatozoa, 0.5 μL of sperm with E400 was directly mixed with a 50 μL drop of activation solution, placed on a glass slide, previously settled on the microscope stage, and immediately after mixing, motility was video recorded under a final magnification of 200× or 400×. Motile spermatozoa were recorded within 10 s for visualization of all spermatozoa. The focal plane was always positioned in the vicinity of the glass slide surface. Video records were obtained with a S-VHS (SONY, SVO-9500 MDP) video recorder at 25 frames/s using a CCD video camera (SONY, SSC-DC50AP) mounted on a dark-field microscope (Olympus BX 50) with a stroboscopic lamp (Chadvick-Helmut, 9630, Ontario, CA, USA) and visualized on a video monitor. The stroboscopic flash illumination with adjustable frequency was set manually to 150–800 Hz depending on the time resolution needed. During the process of recording, the microscope stage was slowly manually moved back and forth: this allowed the visualization of multiple well-defined successive images of a moving sperm without overlap within every video frame [27].

2.6.6. Visualization of Motility of Sperm Flagella by High-Speed Video-Microscopy

Methodology for visualization of fish sperm flagella motility parameters by high-speed video-microscopy was mostly according to Bondarenko et al. [28]. Briefly, in order to observe the detailed pattern of live fish sperm flagella, phase contrast optical microscopy with high magnification (40×–100×) objective lenses was used with oil immersion, resulting in a bright image of the very small diameter flagellum. The high-speed video recording provides high spatial and temporal resolutions (up to several 1000 images/s). Serial frames individually selected from such video recordings allow us to follow successive positions (every millisecond or less) of flagellum waves covering several full beat cycles. Such records allow the description of flagellar images during one or several beat cycles where several successive positions (up to 20) are available for detailed analysis. Analysis of each individual sperm cell image includes quantification of several flagellar parameters such as beat frequency (number of waves developed per second), amplitude and length of the successive waves, curvature of each wave, attenuation factor of waves along the flagellum, and curvature of the general wave pattern [29].

2.6.7. Extender Evaluation by Fertilization, Swim-Up, and Malformation Larvae Rates

Milt from nine AB strain males was individually collected and evenly divided into 20 µL E400 and 20 µL Kurokura (a total of 18 tubes). Only sperm with high motility rate (>90%) in Kurokura and E400 extenders from six males were pooled and used for fertilization. Fertilization experiments were performed after 1.5 h of storage with pooled milt. Prior to fertilization, the concentration of the pooled spermatozoa in the two extenders was as follows: E400−0.063 × 10^9 and Kurokura−0.059 × 10^9 per mL.

Pooled eggs from five females (18 mg, about 80 eggs) were fertilized with 600,000 and 6,000,000 of spermatozoa and replicated four times. Total volume of milt with extenders and hatchery water was always maintained at 100 µL (Table 1). Extenders were added to the water from the hatchery to balance the ions to the level of the first group with a higher sperm concentration. Right after sperm addition, the tubes were shaken by hand for about 45 s. Finally, the fertilized eggs were gently distributed into small cell culture Petri dishes (3.5 cm diameter) filled with dechlorinated water and kept at 25 °C in an incubator (PolyLab, Poland). Each fertilization assay in each extender was replicated three times. The dead (white) eggs were removed at 1 h after fertilization, and the remaining eggs in each Petri dish counted as the initial total number of eggs. The eggs fertilized successfully were counted when embryos reached the 2–4 cells stage at 1.5–2 h after fertilization. Dechlorinated water was gently changed. The dead embryos were removed 24 h after fertilization and then the water was exchanged daily up to completion of swim-up larvae. The swim-up larvae (Figure 1A) and malformed larvae (Figure 1B; unusual body proportions, e.g., peritoneal or heart edema, irregular body axis, and head malformities) were manually counted on day 7 of incubation at 25 °C. For each Petri dish, the fertilization, swim-up larvae, and malformation rates were calculated as the ratio between the number of fertilized eggs or swim-up or malformed larvae relative to the initial number of eggs.

Table 1. Experiment design of fertilization with different pooled sperm numbers (six males) and extenders (E400 and Kurokura) at 1.5 h post stripping (HPS). Total volume of milt with extenders and hatchery water was 100 µL.

Sperm Number per 80 eggs	Extenders	Volume of Sperm without Additional Extenders (µL)	Volume of Additional Extenders (µL)	Volume of Sperm with Additional Extenders (µL)	Volume of Hatchery Water (µL)
600,000	E400	0.95	8.55	9.50	90.50
	Kurokura	1.00	9.00	10	90.00
6,000,000	E400	9.50	0	9.50	90.50
	Kurokura	10.10	0	10.10	89.90

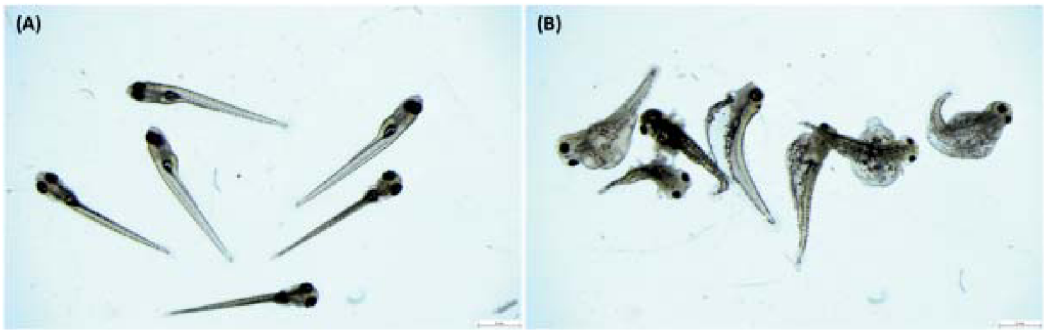

Figure 1. (**A**) Regular swim-up larvae and (**B**) malformed larvae of zebrafish at the age of 7 days (bar scale 1 mm).

2.7. Fourth Step: Testing of Two Different Fertilization Methods

Milt from the AB strain was stripped as described previously and evenly divided into 20 µL of E400 extender and 20 µL of Kurokura extender; only sperm with high motility rate (>80%) from 10 males were pooled and used for fertilization. Fertilization experiments were performed with pooled milt stored on ice for 1.5 h. Prior to fertilization, the concentrations of pooled spermatozoa were 0.058 × 10^9 in E400 extender and 0.067 × 10^9 spermatozoa per mL in Kurokura extender. Sperm was used for fertilization at the same level as before of 6,000,000 spermatozoa per 100 eggs.

First, 1 mL cryotubes were used as small dishes for fertilization (see Video S4). The eggs from six females (22.5 mg, about 100 eggs) were placed at the bottom of the tube and milt as drops added next to the eggs (not on them): (A) with 10 µL milt in E400 extender and activated with 90 µL hatchery water; (B) 9 µL milt in Kurokura extender and activated with 91 µL hatchery water (each time total volume = 100 µL). Second, small Petri dishes (35 mm in diameter and 15 mm in depth) were used for fertilization (see Video S5). The same number of eggs was deposited to the edge of the Petri dishes by pipette tips and then fertilized directly with milt (it was necessary to keep the Petri dish tilted at a 45° angle): (A) 10.27 µL milt in E400 extender and activated with 400 µL of hatchery water; (B) 9.01 µL milt in Kurokura extender and activated with 400 µL hatchery water (total volume was c. 410 µL in each case). The difference between the two methods was that when using cryotubes, the number of spermatozoa in 1 µL of activation solution (water + milt with extender) was 60,000 spermatozoa, and in the case of Petri dishes, only 14,634 spermatozoa. Then the cryotubes and Petri dishes were shaken by hand for about 45 s. Finally, the fertilized eggs were gently distributed into large Petri dishes (9 cm diameter), filled with dechlorinated water, and incubated at 25 °C. Each procedure was replicated three times. Subsequently, incubation took place in a similar manner to the previous experiments.

2.8. Statistical Analysis

The data distribution homogeneity of dispersion was evaluated using Levene's test. All the differences among means were determined by LSD test. The effect of extenders on motility (Figure 2A), VCL, and VSL (Figure 2B,C) at different storage times was performed by two-way ANOVA, respectively. The difference between AB and EGFP strain was tested by independent samples *t*-test, $p < 0.05$. Two-way ANOVA analyses were conducted to find the influence of storage time and individual males on sperm motility, VCL, and VSL (Figures 2 and 3) and the effect of extenders with numbers of spermatozoa or fertilization methods on the fertilization and swim-up larvae rates of success (Figures 5 and 6). The results are presented as mean ± S.E. All analyses were performed at a significant level of 0.05 by using R [30].

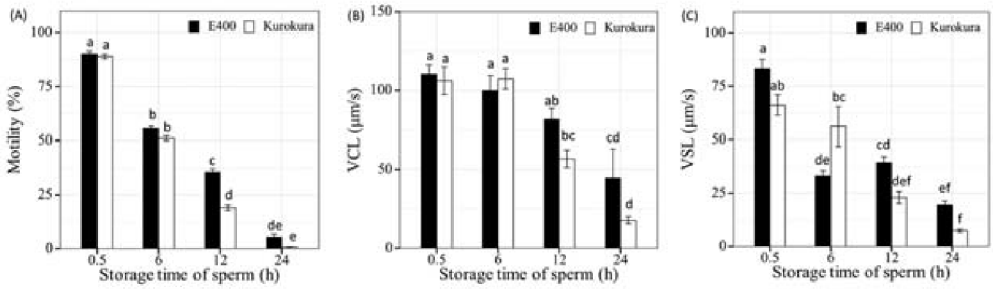

Figure 2. Motility parameters of zebrafish spermatozoa from all nine males at 15 s post-activation after milt storage of 0.5, 6, 12, and 24 h post-stripping (HPS) in Kurokura and E400 extenders: motility rate (%) (**A**); curvilinear velocity (VCL) (µm s^{-1}) (**B**); and straight-line velocity (VSL) (µm s^{-1}) (**C**). Mean ± S.E. with three replications are shown and compared by two-way ANOVA, followed by LSD tests. Groups with a common lower case letter do not differ significantly ($p > 0.05$).

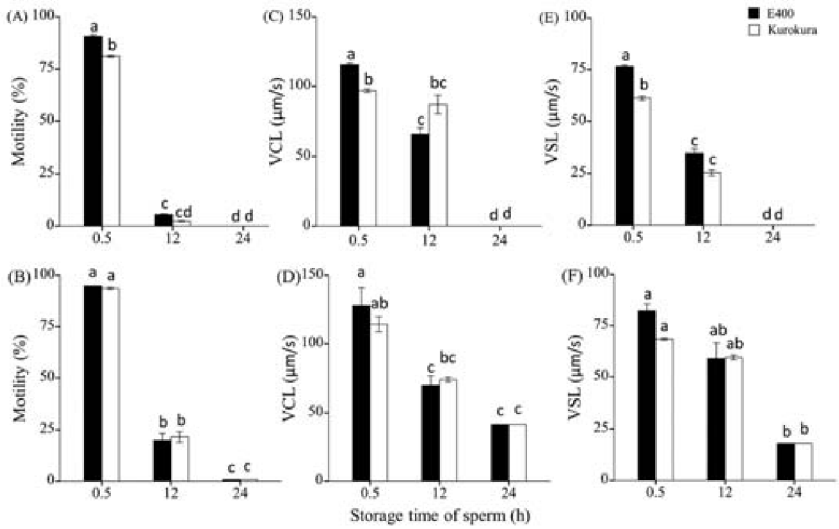

Figure 3. Results with storage of milt from directly pooled sperm (five pooled sperm groups and totally from 45 males) during collection from males (**A**,**C**,**E**) and with individual collection of milt and storage pool of good sperm (**B**,**D**,**F**): motility rate (%) (**A**,**B**); curvilinear velocity (VCL) (μm s^{-1}) (**C**,**D**); and straight line velocity (VSL) (μm s^{-1}) (**E**,**F**) at 15 s post-activation after milt storage of 0.5, 12, and 24 h post-stripping (HPS) in E400 and Kurokura. Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower case letter do not differ significantly ($p > 0.05$).

3. Results

3.1. First Step: Testing "Spontaneous" Movement of Spermatozoa

A drop of milt was collected from a male and then, as soon as possible to avoid it drying up, which could occur within about 10 s, it was directly smeared on the surface of a glass slide so as to observe it under a dark-phase microscope. In all the samples collected from five males, motility of >70% of the spermatozoa was activated. The movement of spermatozoa was forwardly efficient at high speed (over 50 μm/s), not just local vibrations. Due to the high concentration of spermatozoa in a small volume (c. 0.1–1 μL), it was not possible to observe and record sperm movement for a prolonged period.

3.2. Second Step: Testing Extenders

To determine the best extender for zebrafish milt storage, milt from an individual male was collected and added into an extender. Right after mixing milt with an extender, sperm movement was immediately observed under the microscope. The same procedure was applied to the other three extenders. Three extenders, Kurokura, MHBSS, and E400, had a satisfactory sperm inhibition capacity, i.e., the sperm did not move or vibrate after dilution in any of the three extenders. On the other hand, few of the spermatozoa could be activated in HBSS. Sperm placed in Kurokura, MHBSS, and E400 solutions showed, after further activation by transfer in distilled water with storage of 6 h, the highest motility of around 43–65%. After 12 h storage in Kurokura, MHBSS, or E400, then transferred in water, the sperm motility of about 25–46% was recorded. However, there were only a few motile spermatozoa (<1%) observed in the HBSS extender at the same time (Table 2). In summary, it is concluded that Kurokura and E400 showed proportionally stable results after 6 and 12 h with slightly lower standard error than in the case of MHBSS. MHBSS deserves further experimentation, but due to the minimal volume of milt from individual males, it was necessary to choose only two extenders for further testing.

Table 2. Motility (%) of sperm stored in extenders (n = 3) and without extender (control, n = 3) activated by distilled water.

Extenders	Without Activation	Motility (%) (Hours after Activation with Distilled Water)			
		0 h	6 h	12 h	24 h
Control	70% movement	0.0 ± 0.0^{dA}	0.0 ± 0.0^{bA}	0.0 ± 0.0^{bA}	0.0 ± 0.0^{bA}
Kurokura	No movement	80.0 ± 1.7^{cA}	42.5 ± 7.5^{abB}	45.5 ± 3.5^{aB}	1.2 ± 0.2^{abC}
HBSS	<1% movement	87.4 ± 0.5^{abA}	15.4 ± 1.7^{bB}	2.7 ± 0.8^{bC}	0.0 ± 0.0^{bC}
MHBSS	No movement	89.0 ± 0.6^{aA}	49.6 ± 7.0^{aB}	30.3 ± 8.6^{abBC}	6.8 ± 1.6^{aC}
E400	No movement	82.5 ± 1.3^{bcA}	65.0 ± 3.8^{aB}	25.0 ± 5.0^{abC}	0.0 ± 0.0^{bD}

Without activation, observations were made directly after sperm collection (within 10–15 s) under a microscope without activation with distilled water. Data (mean ± S.E.) show the percentage of motile sperm. Different superscript lower case letters (a-d) indicate statistically significant differences between treatments at the same time point (0, 6, 12, and 24 h); different superscript upper case letters (A–D) indicate statistical differences between time points for each treatment (Control, Kurokura, … etc). A one-way ANOVA followed by an LSD test for post hoc multiple comparisons was used for analysis.

3.3. Third Step: Testing of Sperm Storage, Spermatozoa Concentration, Seminal Plasma Osmolality, and Fertilization with Swim-Up Larvae Rates

3.3.1. Storage Sperm from Individual Males

The key quality parameter, motility rate of stored sperm, was measured in nine individual males. The influence of strains on motility parameters was not found. E400 and Kurokura were shown to be suitable extenders to store sperm for fertilization for 6 h at 0–2 °C. Significantly higher motility (16.6%) was observed with sperm stored in E400 than in Kurokura ($p < 0.05$) at 12 HPS. We observed similar differences regarding the VCL and VSL parameters. In total, there were no significant differences of sperm quality parameters (motility, VCL and VSL) between these two extenders at 0.5 and 24 HPS (Figure 2). On the other hand, the effective preservation of sperm movement was relatively low when after 6 h of storage, motility decreased by 35% compared to motility at 0.5 HPS. The difference after 12 h was 55% for the best extender, E400. After 24 h of storage, only 5% of the motile sperm was retained in the E400. The velocity of sperm movement, namely VCL and VSL at time 0.5 HPS, was at a level of 110 and 83 μm/s, and velocity gradually decreased at 24 h storage to 44 and 19 μm/s, respectively, in extender E400.

Two-way ANOVA analysis also showed that storage times and individual males had a significant influence on sperm motility, VCL and VSL, when sperm was stored in E400 and Kurokura extenders. In E400, for some males such as 1 and 4, there was a 10–20% motility rate after 24 h sperm storage (Figure S1A) but the VCL in most of the males (except males 2 and 3) reached 50 μm/s (Figure S1B). However, there was almost no motile sperm in Kurokura at 24 HPS (Figure S2A) and the VCL was only observed in males 1 and 4 at 50 μm/s (Figure S2B). Interestingly, after 0.5 HPS compared to 24 HPS, there was not much difference in VCL velocity. On the other hand, there was a rapid reduction in velocity within VSL. This means that within the VSL, there was a sharp drop in velocity at a straight track (Figure S1B,C). Overall, the E400 proved to be of better efficiency to preserve zebrafish sperm motility when compared to the other extenders.

3.3.2. Storage of Pooled Milt at Time of Collection

The motility of pooled sperm stored in E400 was significantly higher by 10% at 0.5 HPS than that stored in Kurokura. It decreased to <5% at 12 and 24 HPS in both extenders. There was no large variation of VCL between E400 and Kurokura at each storage time, but at 0.5 HPS, VSL decreased more in Kurokura than in E400 (Figure 3C). The VCL and VSL values continued to decrease, reaching 0 at 24 HPS (Figure 3C,E). Two-way ANOVA analysis showed that storage times and extenders had a significant influence on pooled sperm motility and VSL, but extenders had no effect on VCL ($p < 0.05$).

3.3.3. Individually Collected Milt and Storage Pool of Spermatozoa with Good Motility

The motility of pooled good quality sperm in E400 and Kurokura extenders was similar at 0.5 HPS. Then it decreased to around 20% at 12 and 1% at 24 HPS in both extenders. There was no large difference of VCL and VSL values between E400 and Kurokura at each storage time (Figure 3B). The VCL and VSL continued to decrease to 40 and 20 μm/s, respectively, at 24 HPS (Figure 3D,F). Two-way ANOVA analysis showed that storage times but not extenders had a significant influence on sperm motility, VCL and VSL ($p < 0.05$).

The pooled results from individuals producing good quality sperm compared with milt directly pooled on collection showed that the former could be stored for a longer time in extenders. The motility was higher by 20% and VSL by 30 μm/s in pooled good quality sperm than in directly pooled milt during collection from males at 12 HPS. There were 1% motile pooled good spermatozoa with 40 μm/s VCL and 18 μm/s VSL and no motile spermatozoa in directly pooled milt during collection from males at 24 HPS.

3.3.4. Sperm Concentration and Osmolality of Seminal Plasma

The range of spermatozoa concentration per male was 0.08–3.52 $\times$ 10^9 per mL with volume from 0.1 to 2 μL. Sperm concentration had no significant influence on the sperm motility rates, but motility was different with storage time ($p < 0.05$). The range of total sperm quantity collected per male was 13,135–1,905,000 spermatozoa. Osmotic pressure values of diluted seminal plasma were measured as a mean of 44.67 mOsmol/kg, which corresponds to 268 mOsmol/kg as an initial osmolality of the seminal fluid.

3.3.5. Visualization of Sperm Motility with Stroboscopic Light

When spermatozoa were transferred into distilled water, their flagellar motility was immediately activated (Figure 4A). Beating waves propagated along the flagellum usually with three crests of amplitude (one and a half sine wave lengths) at 16 s post sperm activation, as shown in Figure 4A; then, waves started to be slightly dampened in the distal portion of the flagellum, as illustrated at 35 s post sperm activation in Figure 4B. There was only a slight ripple (low amplitude wave) close to the head of the flagellum, indicating only vibration without efficient forward sperm movement at 61 s after sperm activation, as illustrated in Figure 4C. A cytoplasmic droplet was visible at the distal tip of the flagellum, indicating some damage to the flagellum due to the osmotic shock imposed by distilled water (Figure 4C,E). Figure 4D shows a real image of spermatozoa at 16 s post sperm activation without frame distribution at a light flicker frequency of 50 Hz. Finally, Figure 4E shows that at 1 min and 25 s post sperm activation, the spermatozoon was completely motionless.

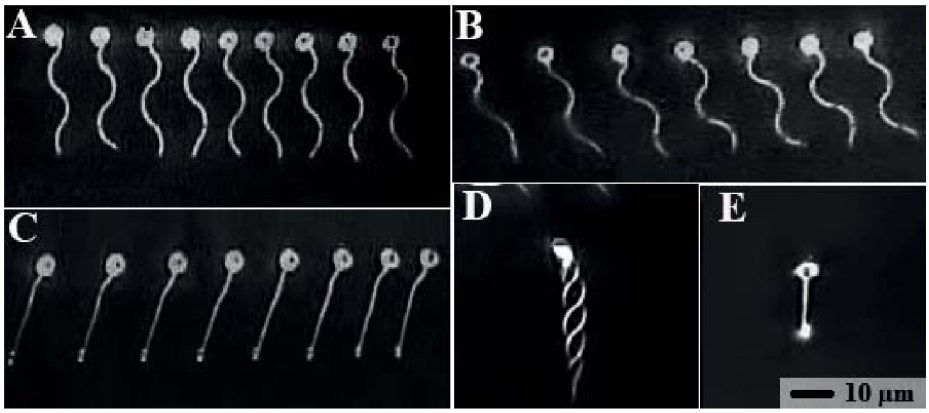

Figure 4. Images of actively swimming zebrafish spermatozoa observed by video microscopy under stroboscopic illumination. Motility was triggered by transfer of spermatozoa from seminal fluid and E400 into the activation solution. Dilution rate of milt with E400 was 1:100. Zebrafish sperm in panels (**A**–**E**), bar scale in (**E**): (**A**) swimming activation after 16 s;

successive images of the same sperm cell illuminated by nine flashes per video frame; (**B**) as (**A**) but 35 s after activation showing dampening of waves in the flagellum (seven flashes per video frame); (**C**) as (**A**) but 61 s after activation shows slightly damaged flagellum (eight flashes per video frame); (**D**) as (**A**) but with overlapping images showing the flagellar envelope (flash frequency = 75 Hz); (**E**) 1 min 25 s after activation, any wave and consequently any swimming has fully ceased.

3.3.6. Visualization of Sperm Motility in High-Speed Video Images

Flagella motility was activated immediately after spermatozoa came into contact with water. Due to technical limitations, the earliest possible video record occurred at 4 to 5 s post mixing. During a time period from 5 to 15 s post-activation, three sine waves with about 5 μm amplitude and 9 μm wavelength were present along the flagellum; the beat frequency (number of waves generated per second) was 48+/− 4 Hz (Hertz or beat/sec) (see Video S6, first part of the clip). Most spermatozoa describe circular tracks of c. 60 μm. As seen in the video images, flagellar waves have a three-dimensional shape, which imposed on the sperm cells a rotation around their progression axis. During a second period from 15 s to 1 min post-activation, sperm flagella continued to develop waves of similar amplitude but localized only in the proximal section closest to the head and absent in the distal flagellum. The flagellar beat frequency decreased to values ranging 15 to 25 Hz, and the sperm tracks became more linear, i.e., large circles (see Video S6, second part of the clip). A third step in the motility period was reached starting from 1 min post-activation. Only a small fraction of the sperm population presented waves of low amplitude and low beat frequency proximally to the head, while the distal $\frac{3}{4}$ of the length of the flagellum was totally devoid of waves and straight. Some blebs can be seen along the flagellum as well as a tail tip curling, both resulting from damages of the flagellar membrane due to the very low osmolarity of water.

3.3.7. Extenders Evaluation by Fertilization and Swim-Up Larvae Rates

The fertilization and swim-up larvae rates were slightly, but not significantly, higher when E400 was used compared to the Kurokura extender (Figure 5). The fertilization and swim-up larvae rates were significantly higher when 6,000,000 spermatozoa were used for fertilization (egg: spermatozoa ratio = 1:75,000) than 600,000 spermatozoa (egg: spermatozoa ratio = 1:7500). The highest fertilization and swim-up larvae rates reached 80% and 40%, respectively. Two-way ANOVA showed that the number of spermatozoa significantly influenced fertilization ($p < 0.001$) and swim-up larvae rates ($p < 0.05$), but different extenders had no significant effect on fertilization and swim-up larvae rates ($p > 0.05$). There were about 1–2% malformation differences between E400 and Kurokura, but the result was not significant.

3.4. Fourth Step, Testing of the Two Different Fertilization Methods

The results (see Figure 6) showed that a tube employed as a container for fertilization and swim-up larvae tests was significantly better than a Petri dish when using milt pre-incubated in E400. Two-way ANOVA showed that the fertilization method and/or spermatozoa concentration in activation water had a significant effect on the fertilization and swim-up larvae rates. Swim-up larvae rate using a test tube showed that E400 had a better swim-up larvae rate than Kurokura. There was about 0–2% malformations in each group without significant difference between E400 and Kurokura.

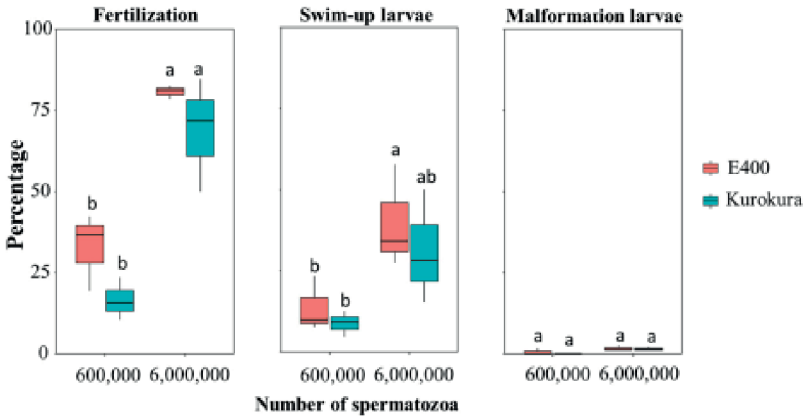

Figure 5. Evaluation of extenders by fertilization and swim-up and malformed larvae level using different amounts and storage times of milt for fertilization. Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower case letter do not differ significantly ($p > 0.05$).

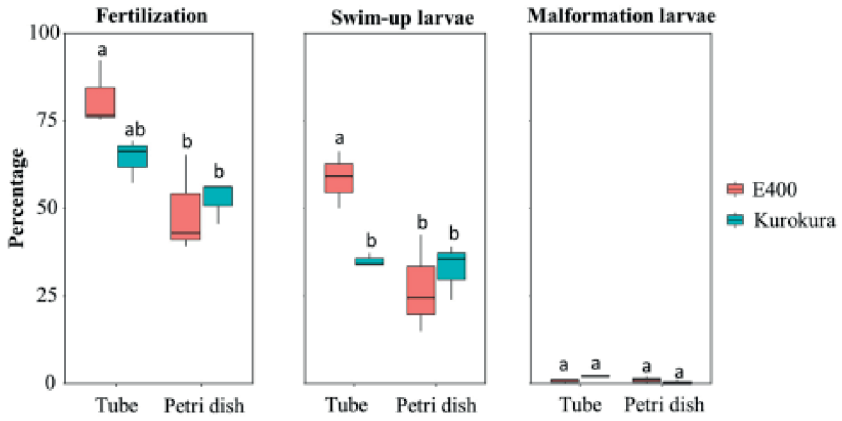

Figure 6. Testing of two test tube and Petri dish fertilization methods by fertilization and swim-up and malformed larvae level using extenders E400 and Kurokura. Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower case letter do not differ significantly ($p > 0.05$).

4. Discussion

4.1. Sperm Motility

The spermatozoa of zebrafish stored in the E400 extender remained immotile, but their motility was activated when transferred to a swimming medium such as fresh water (see Video S6), where they presented a behavior similar to the spermatozoa other teleost species. After activation, they immediately acquired a rapid forward motility; then, after a period of 10 to 20 s, they slowed down gradually until the arrest of flagellum movement. Most sperm stopped moving after 60–90 s of activation. In other cyprinids such as common carp, it is also 60 s [31], and in tench, 40–60 s [15]. A decrease in swimming behavior in most other species could be primarily due to the decrease in energetic compounds (mostly ATP) during the motility period, where there is a trade-off between energy expenditure and motility duration [32]. It should be noted that when the head of the spermatozoon stopped movement, the flagellum continued to vibrate, although it had damaged membranes, especially at the tip.

Spermatozoa of freshwater fishes contaminated with urine showed a similar course of activation during stripping, leading to full arrest if an extender is not used to control the surrounding osmolarity so as to prevent motility. This category of fishes includes zebrafish [18,25,33,34] and the results of the present paper, European catfish (*Silurus glanis*) [21,35], tench [36,37], asp [16], tilapia (*Oreochromis mossambicus*) [38], and marine fishes such as Senegalese sole (*Solea senegalensis*) [39] and turbot (*Scophthalmus maximus*) [40]. In the case of zebrafish, this problem is even more critical because of the very small size of the fish, which makes it difficult to empty the urine from the bladder prior to milt collection (see Video S1). In many fishes, urine accumulates in the bladder and is extruded together with milt during stripping [20,36,39]. It is known that spermatozoa in seminal plasma are immotile, but the hypo-osmotic level of urine changes the environmental conditions of the spermatozoa in the seminal fluid including osmolality, pH, and ion content. The last may spontaneously activate the spermatozoa [41,42]. This spontaneous activation of spermatozoa during the collection of milt destined for artificial fertilization is undesirable because the spermatozoa quickly lose their potency to fertilize [35,43]. All milt samples in our zebrafish study exhibited spontaneously active spermatozoa right after milt collection, because they were activated by contact with urine, evidenced by a mean osmolality value of 268 mOsmol/kg in the urine-contaminated seminal fluid. If milt was not contaminated by urine, the seminal fluid osmolality is predicted to be 288–315 mOsmol/kg, which is the osmolality of blood plasma in zebrafish [13,18]. It was shown that uncontaminated seminal plasma of many fish species presents an osmotic level similar to that of blood plasma [44–46]. Therefore, based on the current study, it is recommended that the urogenital papilla is washed with an extender prior to milt collection (see Video S7), which prevents spermatozoa activation. The milt collected by such a procedure can then be stored in extenders.

Many studies have shown that when spermatozoa are activated by urine contamination, some energetic content is lost within a few seconds before being exposed to an immobilizing solution (IS) [43]. Before sperm is affected by IS, motility parameters change rapidly due to high post-activation energetic consumption. The relationship between motility, respiration, and ATP production was investigated in European catfish [47], common carp [48], and perch (*Perca fluviatilis*) [49]. Usually, half of the ATP was exhausted during the first 5–10 s after activation. This loss of energy can be restored by incubating sperm in IS or in artificial or natural seminal plasma [50,51]. Subsequently, the spermatozoa exhibit increased motility and fertilization capacity; this was demonstrated in common carp [50] and sterlet (*Acipenser ruthenus*) [51]. In summary, a longer storage time of sperm under the optimum osmotic, ionic, and pH conditions will maintain sperm quality and reconstitute energy.

4.2. Milt Storage

To prevent spontaneous movement and extend milt storage time, it was necessary to test the potentialities of extender solutions whose use has been previously published. From four commonly used extenders Kurokura, HBSS, MHBSS, and E400, it was found that the Hanks' balanced salt solution (HBSS) did not stop sperm spontaneous movement. A few spermatozoa moved and vibrated (Table 2). It has been reported that the osmolality of HBSS and MHBSS was 300 and 320 mOsmol/kg [25,26]. We tested the HBSS osmolality and found it to be about 285 mOsmol/kg, which may be the reason the extender was unable to inhibit all movement. However, high osmolality was found in Kurokura, E400, and MHBSS of 370, 400, and 320 mOsmol/kg, respectively. Our results showed that after activation, there were >80% motile spermatozoa after 0.5 h of sperm storage and subsequently 43–65% motility after 6 h and even 25–46% motility after 12 h with sperm stored in Kurokura and E400. This proved that extenders with a higher osmotic pressure, >370 mOsmol/kg, can offset urine contamination and hypotonicity immediately after milt collection and keep sperm cells in an immobilized state. The sperm motility of common carp, another cyprinid, is inhibited by the high osmolality of the seminal plasma [10,52]; concentrations

> 150 mM of KCl or NaCl have been found to inhibit their sperm movement [48,53]. The study by Wilson-Leedy et al. [33] provides crucial information about osmolarity control of zebrafish sperm motility. In this paper, an immobilizing solution was used (called ISS and composed of, in mM: 140 NaCl, 10 KCl, 2 $CaCl_2$, 20 HEPES titrated to pH 8.5 with NaOH; final osmolality = 321 mmol/kg) and a range of osmolarities above and below this value were tested, which provides an important base for the choice of the correct osmolarity of the immobilizing solution. Some of our preliminary results (not detailed in the present publication) confirm the above-mentioned results of Wilson-Leedy et al. [33].

Zebrafish are characterized by a small body size, minute testes, and a minimal milt volume, i.e., <2 µL per male. These characteristics are similar to other small teleost fishes such as Japanese medaka (*Oryzias latipes*) [54], Monterrey platyfish *Xiphophorus couchianus* [55], and green swordtail *Xiphophorus hellerii* [56]. Furthermore, the sperm concentration between zebrafish individuals varied widely in the present study from 0.08 to 3.52 × 10^9/mL, and the total spermatozoa number per male ranged from 13,135 to 1,905,000. Thus, it is necessary to pool milt from at least six males when it is intended to be used for artificial fertilization in the laboratory. Therefore, in the present study, a comparison of milt was made from (1) individual males saved individually, (2) individual collection and preservation of the best pooled sperm, and (3) direct collection and pooling. When the milt was collected from individuals and stored individually, spermatozoa motility decreased at 12 HPS and motility was significantly higher in E400 than Kurokura. There was no difference in VCL and VSL with the two extenders at 12 HPS, but later the VCL and VSL were a little higher in E400 than Kurokura. The preservation of milt did depend on the individual from which it was derived, and sperm from some males lost spermatozoa motility at 6 HPS. Milt individually collected and then pooled with only the best milt was essentially similar to milt stored individually. The worst results were obtained when the milt was mixed directly at collection time from males: the potential spermatozoa motility dropped at 12 HPS to 1–3% in this case. This could be expected, as we mixed good and bad milt, and bad milt probably affected negatively the overall ability to preserve spermatozoa.

It is well known that sperm plasma from bad sperm can significantly decrease the motility rate, VCL, and VSL of good sperm, even when the sperm is kept immotile in the bad seminal plasma [51]. There are many factors such as osmolality, Ca^{2+}, Na^+ ions, some enzymes, and proteins in maintaining sperm quality during storage [51]. Seminal plasma contains many nutrients, among which some are activating or inhibiting components. A similar phenomenon was found in stallions, where adding seminal plasma from low motility sperm (≤20%) to high motility sperm samples progressively reduced sperm motility [57]. As motility is the most important function of the spermatozoon, enabling it to reach the oocyte and fertilize it, simple mixing of milt with the extender E400 immediately at collection is recommended for aquaculture, but only if fertilization is performed relatively soon after, i.e., within about 30 min. In the case where it is necessary to store the milt for a longer period, then only individual male milt stored separately in the E400 solution, or pooled sperm with good motility stored in E400 solution, should be used within a time delay of 6 HPS or up to a maximum of 12 HPS.

4.3. Fertilization and Swim-Up Larvae Rates

In the present fertilization and swim-up larvae rates experiments, >6,000,000 spermatozoa for 80 to 100 eggs with 100 µL of activation solution (c. 90 µL of water + 10 µL milt and extender E400), i.e., a concentration of 60,000 spermatozoa per µL of activation solution, enabled 80% fertilization and 40–60% swim-up larvae rate to be achieved (see Figures 5 and 6). Sperm concentration of 600,000 spermatozoa (egg: spermatozoa ratio = 1:6000) of 1.5 h storage was not successful for fertilization compared to 6,000,000 spermatozoa (egg: spermatozoa ratio = 1:60,000). However, Hagedorn and Carter [1] found that 4 × 10^4 freshly collected spermatozoa was enough (70%) for the fertilization of 30 eggs (egg: spermatozoa ratio = 1:1333). The difference was probably due to the use of sperm aged 1.5 h in the present study. On the other hand, Hagendorn and Carter [1] did not

state the level of hatching. Although in other fish species, such as European catfish, it was found to be better (increased sperm motility, VCL, and VSL when activated and fertilized with hatching level) to store milt for 1 day after stripping or 5 h before freezing in an immobilization solution [20,21,58]. In our study, zebrafish sperm aging is much faster than that in larger fresh- and warm-water fish species such as European catfish and common carp [59]. On the other hand, the preservation values of extenders achieved in zebrafish milt are close to those in another cyprinid, the tench, which also possesses milt easily contaminated with urine and must be collected in an immobilization solution [15].

A Petri dish with a larger volume of water for fertilization is routinely used for zebrafish during fertilization [2]. This traditional method was compared in this study to using a test tube with a volume of water that sufficiently covered 100 eggs. In both cases, the same number of eggs and the same number of spermatozoa were used. The ratio of spermatozoa: egg was also the same in both cases. It was found that there were better fertilization and swim-up larvae rates when using a test tube. This was due to (1) the shape of the containers; (2) the volume of water for fertilization (the test tube volume was four times lower); (3) E400 with milt being diluted 1:10 with water in the test tube and 1:40 in the Petri dish; (4) the osmotic concentration being higher in the test tube than in the Petri dish; and (5) there being a higher spermatozoa concentration in the test tube than in the Petri dish. In the test tube, the E400 extender provided the best hatchability. An optimum ratio of spermatozoa: egg is usually thought to be a key factor in in vitro fertilization [60], but the volume of activation water and number of eggs during fertilization are very important and must always be taken into account [61], as is also evident in the present study. Moreover, the test tube conditions also mimic more accurately the natural reproductive conditions that aquatic animals have adopted through long-term evolution processes [62].

5. Conclusions

In the current study, the movement of the zebrafish spermatozoon in water was ensured by waves (three curvatures) propagated along the flagellum at 16 s after activation. Later on, i.e., around 35 s post activation, the distal half of the flagellum appeared damped (no wave) and finally presented only some ineffective vibrations leading to full arrest. More than 70% of zebrafish sperm samples were activated spontaneously due to contamination by urine. Therefore, for the purposes of sperm preservation and artificial insemination, milt must be immediately mixed with an extender at collection, which fully stops the movement of spermatozoa. In addition, the area of the urogenital papilla must always be washed with an extender prior to sampling (see Video S7). Without it, the fertility of zebrafish sperm cannot be preserved for even a few minutes. The extender E400 allowed storage of sperm for fertilization for 12 h at 0–2 °C. When milt was collected from individual males and stored individually, the sperm motility decreased to 36% at 12 HPS for E400 and to 19% for Kurokura extenders. The motility decreased by 35% at 6 HPS compared to 0.5 HPS. To achieve a sufficient level of fertilization and swim-up larvae rates, a test tube with 6,000,000 spermatozoa, 100 eggs, and 100 µL activation solution has proven to be better than using a Petri dish. Milt stored for 1.5 h in E400 extender at 0–2 °C resulted in the highest fertilization and swim-up larvae rates, 80% and 40–60%, respectively. Finally, it is recommended to use E400 or Kurokura solutions for the practical management of zebrafish.

Supplementary Materials: The following are available online at https://doi.org/10.5281/zenodo.4813606: Video S1: Detail when collecting zebrafish milt, Video S2: Stripping of males with milt collection in E400 and Kurokura, Video S3: Stripping of females, Video S4: Fertilization in a tube, Video S5: Fertilization in a Petri dish, Video S6: Visualization of motility of sperm flagella by High-Speed Video- Microscopy, Video S7: Stripping of males with milt collection in E400. Figure S1: Motility parameters of zebrafish *Danio rerio* spermatozoa from nine individual males at 15 s post-activation after milt storage of 0.5, 6, 12, and 24 h post-stripping (HPS) in E400 extenders: motility rate (%) (A); curvilinear velocity (VCL) (µm s^{-1}) (B); and straight-line velocity (VSL) (µm s^{-1}) (C). Mean ± S.E. with three replications are shown and within each time point using one-way ANOVA, followed by LSD tests. The influence of strains on motility parameters was not found. Groups with

a common lower case letter do not differ significantly ($p > 0.05$). Figure S2: Motility parameters of zebrafish *Danio rerio* spermatozoa from nine individual males at 15 s post-activation after milt storage of 0.5, 6, 12, and 24 h post-stripping (HPS) in Kurokura: motility rate (%) (A); curvilinear velocity (VCL) (μm s^{-1}) (B); and straight line velocity (VSL) (μm s^{-1}) (C). Mean ± S.E. with three replications are shown and compared using one-way ANOVA, followed by LSD tests. The influence of strains on motility parameters was not found. Groups with a common lower case letter do not differ significantly ($p > 0.05$).

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AQUA_2020_1080: Sperm management of European catfish (*Silurus glanis* L.) for effective reproduction and genetic conservation

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ABSTRACT

The European catfish (*Silurus glanis*; also called wels or sheatfish) is one of the largest commercially important freshwater fish species. The objective of this study was to examine the storage time of its semen in an immobilization solution (IS) to enhance conservation and aquaculture programs. Changes in spermatozoan motility was examined in semen stored on ice in 20 mL tubes in IS for 0, 1, 7, 14 and 28 days post-stripping (DPS). Stored sperm samples were pooled and used for fertility testing at 1 and 7 DPS. The IS maintained sperm motility at > 90% during days 1–7 of semen storage, and curvilinear velocity (VCL) and straight-line velocity (VSL) were up to the levels of 163 and 90 μm s^{-1}, respectively. Spermatozoa stored for 14 days had 60% motility and VCL and VSL values were similar to those of 7 DPS. Sperm motility was only 1% at 28 DPS with VCL and VSL of 79 and 24 μm s^{-1}, respectively. The fertilization and total hatching rates of eggs activated with sperm stored for 7 days were reduced, but the slight decrease was compensated by the increase in supply of spermatozoa which resulted in overall 80% level of fertility and hatching. The number of larval malformations was not correlated with storage time of sperm. Based on the results of this study, sperm from the European catfish can be collected and stored in IS for as long as 1 week, retaining quality sufficient for fertilization to yield normal fry.

1. Introduction

The European catfish (*Silurus glanis* L., 1758; also called wels or sheatfish) is a commercially important freshwater fish which naturally occurs from the Aral Sea basin westward to the Danube and Vistula River basins, and southward to Greece and the western part of Anatolia (Banarescu, 1989). It is also likely to appear in some Baltic Sea tributaries and the upper part of the Rhine River. However, most populations and/or strains of European catfish in northern and western Europe are transplanted into non-native areas within the Danube basin (Eurostat, 2017; Linhart et al., 2002; Triantafyllidis et al., 2002). European catfish have been distributed in Europe and have become one of the main targets of recreational anglers on large rivers (Britton et al., 2010; Copp et al., 2009; Cucherousset et al., 2018). It is one of the 20 largest freshwater fish worldwide, attaining a total length 2.7 m and a documented mass of 130 kg (Bouletreau and Santoul, 2016); it is also long-lived with a maximum age in the wild of 80 years (Mihalik, 1982). The meat of this species has a sweet taste and its capacity for growth is among the highest of any fish (Eurostat, 2017). Thus, this catfish is very popular among Europe's sport fishermen as a trophy fish (Lyach and Remr, 2019).

In 2015, the harvest of the European catfish from the total inland fisheries exceeded 11,000 t, where over 65% were caught from rivers in Russia. Inland catches of European catfish in Kazakhstan, the European Union and Turkey were 1500, 750 and 550 t, respectively. World aquaculture production of European catfish today is stable at 1800 t with over 1000 t from the European Union (Eurostat, 2017). To maintain sufficient genetic variability for aquaculture and future well-being of the species, the European catfish is protected by the Bern Convention and the IUCN, classifying it as “Least Concern” in the IUCN red list category and criteria from 2008 (www.iucnredlist.org). An important goal for the countries in which they live naturally is to maintain or recover natural stocks and protect critical habitat (Zibiene and Zibas, 2019). Therefore, conservation programs for isolated

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European populations of *S. glanis* should be undertaken. In the Czech Republic, live gene resources of Bohemian populations of European catfish are maintained *in vivo* and *in vitro* at a hatchery as is a bank of cryopreserved sperm (Flajšhans et al., 1999; Linhart et al., 2005).

Normally in natural condition European catfish is spawn in pairs and spawning can be promoted in ponds or rivers. European catfish makes a nest in the roots of willow trees or on the vegetation that is available to which the stripped eggs stick. Spawning is nocturnal. Following the spawning the male guards the nest for several days, and makes sure, that the eggs are well ventilated by repeatedly fanning his tail fin (Horvath et al., 1992; Copp et al., 2009). The movement of European catfish within Asia and Europe has been facilitated by the development of techniques for artificial reproduction (Fijan, 1975; Horvath and Tamas, 1976; Krasznai et al., 1980; Kouril et al., 1995), but significant problems in propagation persist. For artificial propagation, male catfish must be killed to collect testicular semen because only small quantities of semen can be obtained by stripping. Testes is a paired organ which is folded several times within abdominal cavity (Mananos et al., 2009). That is probably the main reason why we get small amount of sperm. The volume of sperm is always greater if the male is very well anesthetized (Linhart et al., 2004). Also, semen collected by stripping is contaminated with urine which results in activation of spermatozoa and a loss of motility within 2 min (Linhart et al., 1987). Furthermore, egg stickiness complicates the process. Gonadotropin injections have increased semen production, the use of an immobilization solution (IS) has somewhat resolved the semen contamination (Kouril et al., 1995; Linhart et al., 1987; Linhart and Billard, 1994; Linhart et al., 2004; Saad and Billard, 1995), and the use of alcalase enzyme has successfully eliminated the problem of egg stickiness (Horvath, 1977, 1980; Legendre et al., 1996; Linhart et al., 2004). However, additional improvements can be realized by short-term storage of semen. Therefore, evaluation of currently used techniques for sperm storage is needed. It has been demonstrated that an artificial extender can prolong the storage time and maintain sperm motility in common carp, *Cyprinus carpio* L. (Cejko et al., 2018, 2019). Low quality common carp sperm can be revitalized by incubation in an artificial seminal plasma composed of $CaCl_2$, Mg_2SO_4, Tris, NaCl, KCl with 310 mOsm kg^{-1} (Cejko et al., 2019). It means that it is possible to enhance low quality/immotile sperm by exposure to a species-specific artificial solution.

European catfish broodstock are usually selected in May–July (Linhart and Billard, 1995) and for induced spawning, hormonally treated with carp pituitary (CP) or ovopel (LHRHa, D-Ala^6 metoclopramide manufactured in Hungary). Females ovulate after one or two CP doses of 4–5 mg kg^{-1} b.w. (Fijan, 1975; Horvath and Tamas, 1976) or ovopel in similar dosages to CP (Brzuska, 2001). Males spermiation occurs after injection of CP at 5 mg kg^{-1} b.w. (Linhart et al., 2004). Semen is always contaminated by urine during sperm collection, resulting in unwanted activation (Linhart et al., 1987). If semen is stripped into an IS (137 mM NaCl, 67 mM KCl, 133 mM glycine; Linhart et al., 1987) sperm remain immotile. Today, sperm is routinely stripped into an IS of 200 mM NaCl, 30 mM Tris-HCl, pH 7 (Linhart and Billard, 1994; Saad and Billard, 1995) and stored for 48–72 h at 4 °C (Linhart et al., 2004). Spermatozoa are added to ovulated eggs and immediately flooded with an activating solution (Horvath, 1977; Linhart et al., 2004). The stickiness of eggs is eliminated with a clay or talc suspension added to the fertilized eggs (Kouril et al., 1995), and with alcalase enzyme solution after 10–12 h of incubation (Horvath, 1977, 1980), or directly after fertilization using proteolytic trypsin or alcalase enzyme (Legendre et al., 1996). Fertilized eggs are incubated in Weiss jars and hatching is expected 2.5–3 days (60 degree-days) after fertilization at 22–23 °C (Linhart et al., 2004).

European catfish from domesticated stocks have less genetic diversity than fish from wild populations, possibly due to passive selection under hatchery conditions (Krieg et al., 2000). Therefore, in future artificial propagation, an increased number of males and females should be used. Despite the fact that this method of reproduction was put into practise before 2000, it is now only used in Central Europe and France.

The aim of this study was to determine the optimum storage time for European catfish semen in IS (Linhart et al., 2004), the quantities of semen necessary to assure at least 80% fertilization and hatching, and to estimate any associated post-hatching malformations in the larvae.

2. Materials and methods

All experiments were conducted according to the principles of the EU-harmonized Animal Welfare Act of the Czech Republic. Manipulations with animals were performed according to authorization for breeding and delivery of experimental animals, reference number: 56665/2016-MZE-17214 (17OZ19180/2016-17214), valid for 5 years from 4 October 2016, issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia by the Ministry of Agriculture of the Czech Republic.

2.1. Broodstock European catfish handling and gamete collection

The research was carried out at the experimental station of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. The broodstock was cultivated and prepared for reproduction according to Linhart et al. (2002, 2004). Males and females suitable for stripping were selected in May–July and kept isolated in 4600 L tanks divided into individual compartments with a water flow rate of 0.2 L s^{-1} at 22–23 °C. Before injection and gamete collection, fish were anesthetized in a solution of 2-phenoxyethanol (1 ÷ 1000).

Ten females were injected and eggs from six were used for two fertilization experiments (mean age and wet body weight: 13 years and 14.5 kg). Female brood fish were acclimated for 1 day before being intramuscularly injected with carp pituitary (CP) dissolved in 0.9% (*w/v*) NaCl solution; administered dosage was 5 mg kg^{-1} b.w. Ova were collected gently at 500–510 degree-hours (degree-hours = sum of temperature at each hour) post-stimulation in plastic bowls. In each fertilization assay, good quality ova from individual females were chosen visually (dark color without urine), stored at a temperature of 18–20 °C and used within 1 h after collection.

Ten males were injected and sperm from six were used for all experiments (mean age and wet body weight: 13 years and 21 kg). They were injected with CP at a dosage of 5 mg kg^{-1} b.w. 24 h before stripping at 22 °C. Sperm was collected from each male by a plastic extractor (4 mm diameter) connected to a vacuum pump and kept in 20 mL tubes containing IS (200 mM NaCl, 30 mM Tris-HCl, pH 7; Linhart et al., 2004). Eight mL of semen with urine was collected into 10 mL of IS. During collection of semen with urine, the tube was shaken rapidly to mix the IS with semen. The IS stopped the sperm movement caused by the urine. Finally, the tube was closed leaving 2 mL of air above the 18 mL mixture of IS, semen and urine (the ratio of air:-sperm = 1:9). Diluted sperm from individual males was stored in a refrigerator on ice at 0–2 °C and pooled prior to each fertilization test.

2.2. Experimental design

Semen samples from three males were stored in 20 mL tubes on ice in IS for 0, 1, 7, 14 and 28 days post-stripping (DPS). Stored sperm from three males were pooled and used for testing fertility and hatching at 1 and 7 DPS.

For testing of sperm fertilization on the first DPS, ova from three freshly spawned individual females were individually used (not pooled). The same three males as in the first case were used to collect control fresh semen for fertility testing together with the first day of semen storage.

For testing of sperm fertilization on the seventh DPS, the ova from

three freshly spawned individual females were individually used (not pooled) and pooled semen from three fresh males was used as a control.

Fertilization tests were not conducted for 14 and 28 days of sperm storage.

2.3. Spermatozoa motility and velocity assessment

Motility was initiated by diluting sperm samples at a ratio of 1:40 in an activation medium [17 mM NaCl + 5 mM Tris–HCl buffered at pH 8.0, which contained 0.25% Pluronic F-127 (to prevent spermatozoon from adhering to microscope slides)]; samples were examined microscopically under a 10× lens, negative phase-contrast condenser with ISAS digital camera (PROISER, Spain) equipped with uEye Cockpit software setting at 25 frame s^{-1} after 0, 1, 7, 14 and 28 DPS. Video records were saved in AVI format and analyzed by the Integrated System for Semen Analysis software (PROISER, Spain) at 15, 30 and 45 s post-sperm activation. Computer-assisted sperm analysis (CASA) measured spermatozoon curvilinear velocity over the actual path (VCL, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}) and percentage of motile spermatozoa (motility rate, %). Analyses were carried out in triplicate and VCL $<$ 10 μm s^{-1} was considered as non-motile. All observations were performed at room temperature (21 °C).

2.4. Fertilization of eggs, incubation and hatching

Fertilization experiments were performed at 1 and 7 days of sperm storage. Prior to fertilization, the concentration of pooled sperm per mL was as follows: short-time storage sperm 0.92 × 10^9, control sperm used 1 DSP 0.94 × 10^9 and control sperm used 7 DSP 0.65 × 10^9. A Burker cell hemocytometer was used to determine spermatozoan concentration (Marienfeld, Germany) (12 squares counted for each male) under an Olympus microscope BX 41 (4009) and expressed in 10^9 mL^{-1}.

Before the fertilization experiments, sperm with IS were again diluted in IS (Linhart et al., 2004). The first DPS, the ova (0.30 g, about 55 ova) were inseminated by pipetting 1-day-old sperm at a level of 2.72 μL = 50,000 spermatozoa and 27.72 μL = 500,000 spermatozoa (dilution 20 μL sperm +980 μL IS) and with fresh control at a level of 2.66 μL = 50,000 spermatozoa and 26.6 μL = 500,000 spermatozoa (dilution 20 μL sperm +980 μL IS). Seven DPS, the ova (0.30 g, about 55 ova) were inseminated by pipetting 7 day old sperm at levels of 27.2 μL = 500,000 spermatozoa and 5.43 μL = 5,000,000 spermatozoa (without additional dilution) and with fresh control at levels of 19.23 μL = 500,000 spermatozoa and 7.69 μL = 5,000,000 spermatozoa (without additional dilution).

Sperm was not added directly to the ova, but the pipette was inserted to the bottom of the 25 mL beaker next to the eggs. Each group was replicated four times, therefore, after sperm addition there were four beakers which were placed on a shaking table at 22 °C. The mixing table was turned on for 1 min (150 rpm) and 3 mL of water was pipetted into each beaker. Then, the fertilized eggs were gently distributed into four glass Petri dishes (eggs from each beaker were placed into a separate Petri dish). Each Petri dish was filled with dechlorinated water. The 95 mL Petri dishes were 9 cm in diameter and 1.5 cm in depth.

Each group of four Petri dishes were placed in four plastic boxes (13.5 cm × 10 cm × 6.5 cm) filled with dechlorinated water (300 mL) which in turn were placed in one larger plastic box (28 cm × 21.5 cm × 7.5 cm; Cheng et al., 2020). These boxes were kept in an air-conditioned laboratory at 22 °C. The eggs in each Petri dish were counted and recorded as the initial total number of eggs. Dechlorinated water was gently changed in the small plastic boxes at 48 h post-fertilization and non-developing embryos were removed; then water was exchanged daily up to hatching. The eye-stage embryos were counted as fertilized eggs at 48 h post-fertilization; the hatched larvae and malformed larvae were manually counted directly after hatching, usually about 4 days of incubation at 22 °C. For each Petri dish the fertilization rate was calculated as the ratio of eye-stage embryos at 48 h post-fertilization to the initial number of eggs, the hatching rate was calculated as the ratio of all hatched larvae to the initial number of eggs and the malformation rate was calculated as the ratio of malformed larvae to the initial number of eggs.

2.5. Data evaluation and statistical analysis

The sperm motility, VCL and VSL at 15 s, 30 s and 45 s post-sperm activation were evaluated as the effect of sperm storage time for individual males. Fertilization, total hatching and malformation rate were evaluated as the effect of sperm storage time, number of spermatozoa per 55 eggs in 3 mL of hatchery water with female effect.

The sperm motility parameters and fertilization success following sperm storage time were analyzed using a multiple ANOVA. The data distribution homogeneity of dispersion was evaluated using Levene's test. All the differences among means were determined by an LSD test. The results are presented as mean ± S.D. All analyses were performed at a significant level of 0.05 by using R (R Core Team, 2018).

3. Results

3.1. Sperm motility parameters

The quality parameters (motility rate, VCL and VSL) of stored sperm in IS varied significantly ($P < .05$) according to storage time (Figs. 1–3). Surprisingly, we found that the motility rate, VCL and VSL of spermatozoa increased somewhat after 1 day of storage compared to results just after sperm collection. Moreover, all three parameters

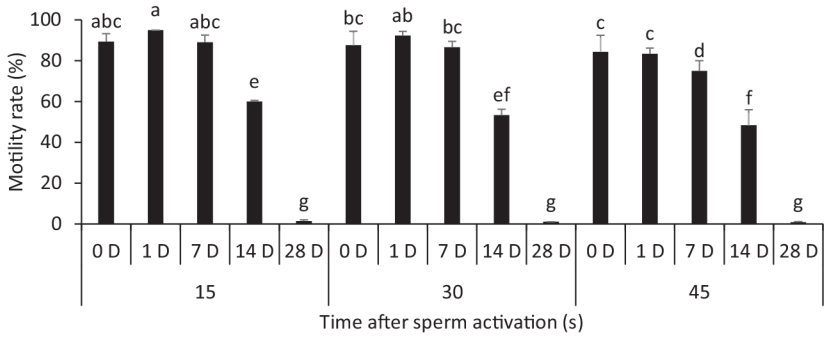

Fig. 1. Motility rate (%) of European catfish *Silurus glanis* spermatozoa at 15, 30 and 45 s post-activation after sperm storage of 0, 1, 7, 14 and 28 days post-stripping (DPS). Data are expressed as mean ± SD; values ($n = 9$) with a different letter are significantly different ($P < .05$).

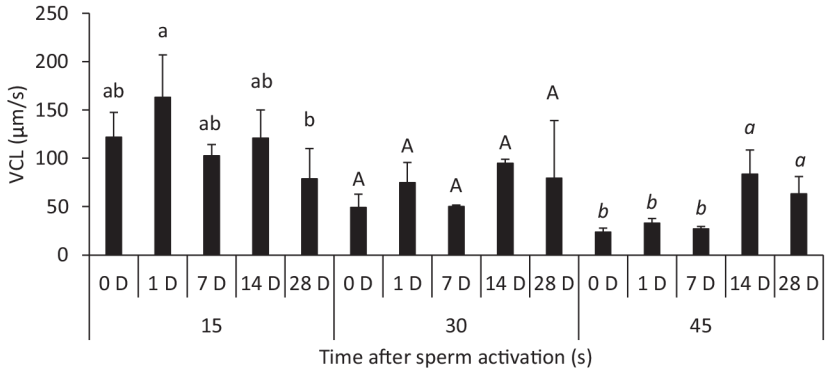

Fig. 2. Curvilinear velocity (VCL) (µm s^{-1}) of European catfish *Silurus glanis* spermatozoa at 15, 30 and 45 s post-activation after sperm storage of 0, 1, 7, 14 and 28 days post-stripping (DPS). Data are expressed as mean ± S.D.; values (n = 9) with a different letter are significantly different ($P < .05$).

showed similar values after storage of 0 and 7 days. Multiple analysis showed that storage times and different incubation groups of sperm had significant influence on sperm motility, VCL and VSL. Overall, the IS proved to be very effective in preserving European catfish sperm motility. In days 1 to 7 of semen preservation, the motility rate was higher than 90% and VCL and VSL were up to a level 163 and 90 µm s^{-1}, respectively. Even after 14 days of storage, semen retained its vitality, with motility reaching 60% and VCL and VSL showing similar values to those after 7 days of preservation. Following a longer storage time of 28 days, a rapid reduction occurred in sperm motility rate to 1%, while VCL and VSL still had values of 79 and 24 µm s^{-1}.

3.2. Fertilization and hatching success

The fertilization and total hatching rates of sperm after 7 days of storage were significantly lower than sperm stored for 1 day, when 500,000 spermatozoa were used for fertilization (egg: spermatozoa ratio = 1:9090). On the other hand, after 7 days of sperm storage, fertilization and hatching rates decreased. However, by increasing the number of spermatozoa the decline was compensated for. Ten times the number of sperm stored for 7 days (5,000,000 spermatozoa; egg: spermatozoa ratio = 1:90,909) achieved the same fertilization and hatching rates as the control fresh semen (Fig. 4 A and B). The post-hatching malformations were usually similar to fertilization and hatching levels of 5–15% (Fig. 4C). The level of larval malformation was not significantly correlated to the sperm storage time (days) but was related to the level of fertilization and hatching rates with final values of 80% fertility and total hatching. In addition, regression analysis confirmed a high correlation of fertilization and hatching rates with storage time of sperm. On the other hand, the storage time of sperm did not correlate with the level of malformations of the fry (Fig. 5). Multiple analysis showed that both storage time and sperm quantity had a significant influence on fertilization and hatching rate.

4. Discussion

The motility of European catfish sperm normally lasts for only 1 to 2 min. The urine osmolality is the key factor causing spontaneous activation of the spermatozoa. This movement is triggered by the decrease of osmolality of urine, which is eight times lower than the seminal fluid, thus urine contamination has a negative effect during artificial propagation by activating spermatozoa prematurely (Linhart et al., 1987, 2004). The use of IS (200 mM NaCl, 30 mM Tris-HCl, pH 7) (Linhart et al., 2004) retained the potential for sperm motility at 60% for 14 days. Semen stored in IS for 7 days, produced fertilization and hatching rates of 20% when used at a ratio of egg: spermatozoa of $1:10^4$, but by increasing the ratio to $1:10^5$ fertilization and hatching increased to 80%. While these positive results for the European catfish were published earlier (Linhart and Billard, 1994; Saad and Billard, 1995), it was recommended that semen should be stored in IS for 2–3 days at 4 °C. However, we think that the greater success, demonstrated in the present study, was probably because the semen and IS mixture was kept on ice at a temperature < 4 °C. The semen was also kept in a closed 20 mL tube with a ratio of air: semen = 1:9 where it

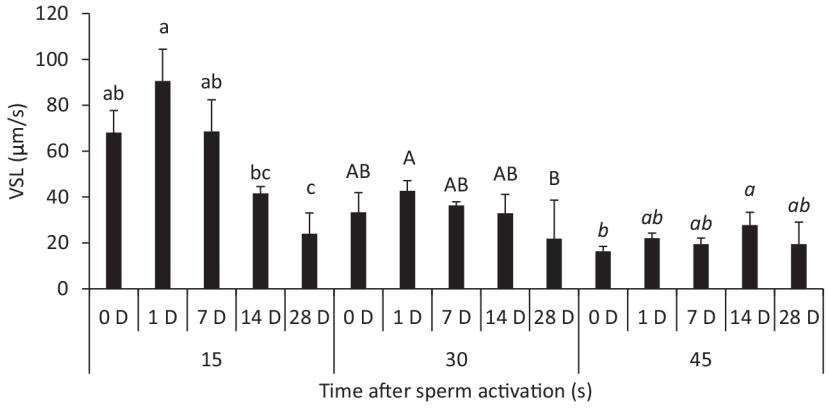

Fig. 3. Straight line velocity (VSL) (µm s^{-1}) of European catfish *Silurus glanis* spermatozoa at 15, 30 and 45 s post-activation after sperm storage of 0, 1, 7, 14 and 28 days post-stripping (DPS). Data are expressed as mean ± S.D.; values (n = 9) with a different letter are significantly different ($P < .05$).

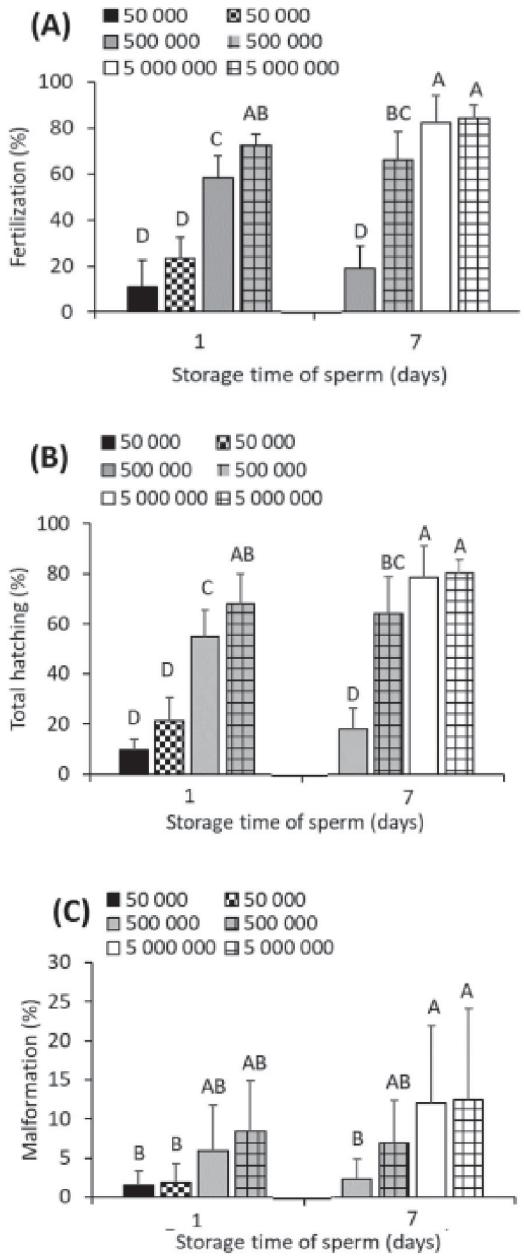

Fig. 4. Fertilization (A), total hatching (B) and malformation (C) rates (%) in European catfish *Silurus glanis* after storage of sperm 1 and 7 days post-stripping (DPS). Comparisons are made with fresh sperm as a control (columns with internal grid and checkerboard). Sperm for fertilization was used in different number of spermatozoa. Data are expressed as mean ± S.D.; values (n = 12) with a different letter are significantly different (P < .05).

therefore had a lower oxygen level. Also, we tried to minimize bacterial contamination and other microflora by vacuum-pumping the semen directly into IS; the closed tubes also limited subsequent contamination. The positive effect was that even after 7 days we did not detect the development of bacteria using a microscope, which usually accompanies the preservation of semen after 3–4 days (Linhart et al., 2004). A primary component of the IS is a Tris-HCl buffer; in biochemistry and molecular biology, Tris-acetate-EDTA and Tris-borate-EDTA buffers are widely used. A number of enzymes are inhibited by Tris, and therefore it should be used with care when studying proteins (Desmarais et al., 2002; Ghalanbor et al., 2008). Furthermore, Tris also increases of the cell membrane permeability (Irvin et al., 1981). These are probably the reasons why bacteria colonize stored sperm very slowly, and the "mild" toxicity of the buffer did not affect the sperm of European catfish.

European catfish sperm stored for 1 day in IS had a slight increase in spermatozoa motility rate, VCL and VSL compared to motility parameters for freshly collected sperm. These three parameters had a similar value between day 0 and 7 days of storage. A similar phenomenon was demonstrated by Linhart et al. (2005), where the storage of European catfish sperm in IS for 5 h before freezing, increased fertilization and hatching rates of post-thawed sperm, and increased percentages of sperm motility and live spermatozoa. We assume that when spermatozoa are activated by urine contamination, some energetic content is lost within the few seconds before being exposed to IS (Alavi et al., 2019). Before sperm is affected by IS, all motility parameters change rapidly in accordance with the high post-activation energetic consumption. The relationship between motility, respiration and ATP production was investigated for European catfish by Billard et al. (1997), common carp (*Cyprinus carpio* L., 1758) (Perchec et al., 1995) and perch (*Perca fluviatilis* L., 1758) (Boryshpolets et al., 2009); usually 50% of ATP was exhausted during the first 5–10 s following activation. This lost energy can be restored within minutes by incubating sperm in IS, or in artificial or natural seminal plasma. Subsequently the spermatozoa exhibit increased motility and fertilization capacity; this was demonstrated in common carp (Linhart et al., 2008) and sterlet (*Acipenser ruthenus* L., 1758) (Xin et al., 2020). Finally, a longer storage time under the optimum osmotic, ionic and pH conditions will maintain quality and reconstitute energy, if there is no contamination by microflora.

The stripping of male European catfish is still not widely practiced in Europe except for the Czech Republic and France (Linhart et al., 2000). Prior to egg collection, usually two to three males are killed, and testicular sperm is extracted from the testes. The scant volume of semen obtained from stripping European catfish is one of the reasons that males are killed during propagation. In our experiment, we collected a total of 3 × 18 mL of semen with a concentration of 0.92 × 10^9 sperm from three males, which provided about 49.68 × 10^9 spermatozoa. According to estimates from the practical reproductive methods practiced in the Czech Republic and France, using semen stored for 1 day should provide a quantity sufficient to fertilize 14 kg of European catfish eggs (2.5 × 10^6 ova; ratio egg:spermatozoa = 1:2 × 10^4) (Linhart et al., 2004). If semen is stored for 7 days, around 10 times more semen would need to be used, thus fertilizing 1.4 kg of eggs (0.25 × 10^6 ova; ratio egg:spermatozoa = 1:2 × 10^5). These calculations are estimated based on an 80% of the hatching level (Fig. 4) and the sperm quantities that were used.

Sperm storage results in a reduction in quality for sperm motility and velocity, seminal plasma composition, and changes in oxidant and antioxidant levels (Billard et al., 1995; Kidd et al., 2001; Linhart et al., 2003; Shaliutina-Kolešová et al., 2014). Moreover, sperm maturation followed by aging processes, results in a decrease of energy stores, DNA integrity, protein changes and fertilizing ability of fish spermatozoa (Dreanno et al., 1999a, 1999b; Shaliutina et al., 2013a, 2013b; Zietara et al., 2004). Fertilization success, embryo quality and later performance of the offspring are highly dependent on the integrity of the gametes. It is known that environmentally induced changes in gametic phenotype have implications for fertilization level and offspring fitness (Alavi et al., 2008; Crean and Bonduriansky, 2014; Gasparini et al., 2017; Zajitschek et al., 2014). These findings are important, because variance in offspring fitness transmitted solely *via* sperm by breeding designs will be attributable to the additive genetic variation. For the time being, the effect of short-term semen storage at 4 °C on the appearance of embryonic malformations has not been demonstrated, but different abnormalities have been correlated with semen from males that have been exposed to pesticides or other toxic compounds (Labbé

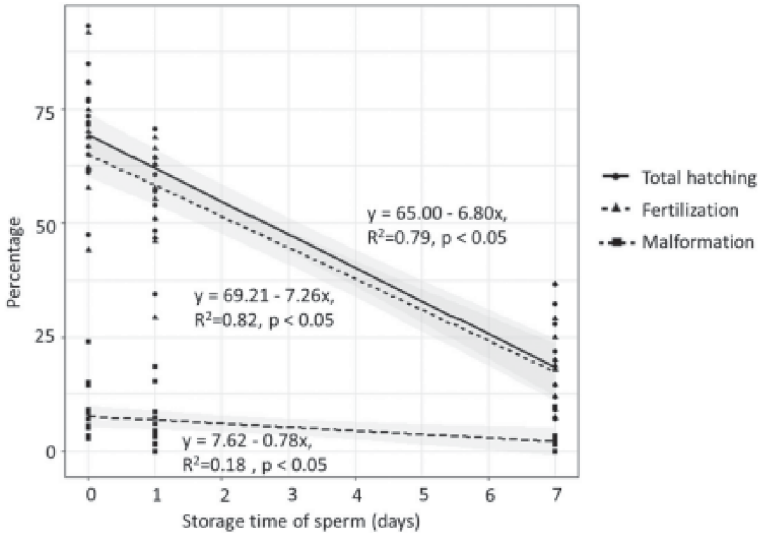

Fig. 5. The relationship of regression values between total hatching, fertilization and malformation, and storage time of sperm 0, 1 and 7 days post-stripping (DPS) in European catfish *Silurus glanis*. The shaded color near the lines are the 95% confidence limits.

et al., 2017). In our case, the dependence between semen aging and the level of embryonic malformations was not shown. Apparently, the DNA of European catfish sperm after a week of preservation was resistant and/or eggs were able to repair damaged sperm DNA; Kopeika et al. (2004) demonstrated this phenomenon in loach [*Misgurnus fossilis* (L., 1758)] and Fernandez-Diez et al. (2016) in rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)].

Based on the results in the present study, we suggest that a practical quantity of sperm from European catfish males can be collected and stored in IS for a week while maintaining good fertilization capacity. This approach allows even small hatcheries to manage broodstock efficiently. Reproduction can be divided into two stages: stripping of males, then 2–3 days later collecting eggs. European catfish are aggressive in confinement therefore fish must be kept separated (Kouril et al., 1995; Linhart et al., 2004). In the past and in some countries where animal protection legislation has not been developed, injuries from aggression has been reduced by closing the fish's mouth with twine after drilling a hole in the jaw (Horvath et al., 1992). This method probably interferes with respiration and of course, is contrary to EU legislation.

As already mentioned in many countries of Europe and Asia, only a few males are used to reproduce the European catfish, this practice is contrary to the Bern convention for protecting and maintaining genetic diversity. Protecting the resource is an important goal for countries in which they live naturally (Zibiene and Zibas, 2019). Low genetic variability was reported about 25 years ago in stocks reared in Europe (Krieg et al., 2000; Triantafyllidis et al., 1999). They explained this phenomenon as the "founder effect" since the natural populations (Volga and Danube Rivers) from which the stocks were derived were subject to passive selection under hatchery conditions, despite the fact that the originated stock was from the most variable European populations (Flajšhans et al., 1999; Krieg et al., 2000; Triantafyllidis et al., 1999). Today, genetic variability is narrowing, as evidenced by a genetic study in Sweden (Palm et al., 2019). In order to minimize generational inbreeding, the effective breeding number (Ne) of parental populations should be at least 50 mature individuals (FAO, 1981). Therefore, conservation programs for isolated European populations of European catfish should be undertaken, and the use of short-term semen storage is one of the tools that can facilitate the implementation of such conservation programs.

5. Conclusion

Spermatozoa of the European catfish stored in IS for 14 days had 60% motility and velocity was similar to those of 7 days post-stripping. The positive effect of IS was that even after 7 days we did not detect the development of bacteria, which usually accompanies the preservation of semen after 3–4 days. The fertilization and total hatching rates of eggs activated with sperm stored for 7 days were reduced, but the slight decrease was compensated by the increase in supply of spermatozoa. The dependence between semen aging and the level of embryonic malformations was not shown.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Improving motility and fertilization capacity of low-quality sperm of sterlet *Acipenser ruthenus* during storage

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ABSTRACT

Improvement of sperm quality with low motility by storage could ensure higher success of fertilization and maintain higher genetic diversity, especially for sturgeons, which as endangered species have limited broodstock and gametes. Sperm was collected from mature male sterlet *Acipenser ruthenus* and motility was evaluated using the CASA system; samples were categorized as GS 'good sperm' (>80%) or BS 'bad sperm' (<20%). Samples from both groups were incubated with seminal plasma from good- (GSP) and bad-quality sperm (BSP), respectively for 15 min, 6 h, 24 h and 96 h at 4 °C. Motility of BS incubated in GSP increased after different storage times compared to BS incubated in BSP, while the motility and velocity of GS incubated in BSP decreased compared to GS incubated in GSP. Fertilization rates were evaluated with samples stored for 15 min and 6 h post-stripping; fertilization and hatching rate of BS after incubation in GSP increased significantly compared to the BS incubated in BSP. Inorganic ion (Na^+, K^+, Cl^-) concentrations and osmolality of BSP were significantly lower than that of GSP. These results indicated that sterlet sperm quality can be revitalized by incubation with GSP. Further, fertilization capacity of BS after incubation in GSP can reach similar levels to the good quality sperm (~70%). Low ion concentration and osmolality in BSP may be a partial cause of low sperm quality. The current study is the first report on the capability to revitalize low quality sterlet sperm by storage in GSP.

1. Introduction

Fish culture is being developed throughout the world to reduce fishing pressure on natural populations. High quality gametes are required to ensure successful artificial reproduction. However, the quality of sperm obtained from captive males can differ significantly between individuals. In sturgeons, during the spawning season, it is possible to obtain good quantities (up to 100 mL per male) of sperm, but the quality is poor [1]. Low quality sperm can be a function of seasonality where different males are at various levels of maturity, but can also be a result of aging of the sperm [2,3]. Low quality sperm reflects poor structure or morphology and altered physiology [4,5]. The alteration of the surrounding ionic composition, pH and osmolality, as well as organic components of seminal plasma has been shown to decrease sperm quality in terms of motility, velocity and fertilization capacity [4,6–8]. Seminal plasma with a stable level of osmolality, ion concentrations, pH and proteins provide an optimal external environment which can protect spermatozoa viability and fertility, and also be indicative of gamete quality [8,9]. On the other hand, seminal plasma with low osmolality or ion concentrations can induce spermatozoa motility prior to fertilization. In addition, several authors have concluded that male fertilization potential is also affected by inorganic and organic components, including protein composition of the seminal plasma [8,10–14].

It has been demonstrated that an artificial extender can prolong the storage time and maintain sperm motility in the Gulf sturgeon *Acipenser oxyrinchus desotoi* and the shortnose sturgeon *Acipenser brevirostrum* [15]. Testicular sperm from the sterlet *Acipenser*

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ruthenus will not become motile until it has been mixed with urine, or incubated in seminal plasma or an artificial solution [16]; the mixing of testicular sperm with urine appears to be essential in spermatozoa maturation of sturgeons and is important in maintaining fertility. The seminal plasma from Wolffian ducts and urine in sturgeons have similar ion composition and osmolality of 32–34 mOsmol/kg. Nevertheless, the ion compositions and osmolality of the testicular sperm (250 mOsmol/kg) are quite different [16].

Spermatozoa of rainbow trout *Oncorhynchus mykiss* incubated in an artificial sperm-motility-inhibiting-saline solution, which contained seminal plasma proteins or pure seminal plasma had, significantly higher motility and velocity after activation than that stored in artificial sperm-motility-inhibiting-saline solution without seminal plasma. These results indicated that seminal plasma proteins have a physiological function which prolongs and stabilizes sperm viability [17]. Additionally, low-quality common carp *Cyprinus carpio* sperm can be revitalized by incubation in an artificial seminal plasma composed of $CaCl_2$, Mg_2SO_4, Tris, NaCl and KCl with 310 mOsmol/kg [18]. Thus, it is possible to enhance low-quality and immotile sperm by exposure to a species-specific artificial solution, and osmolality has a protective effect relative to temperature changes and from the toxic action of cell metabolism, as well as securing appropriate gaseous exchange during storage.

Success of artificial reproduction depends on a sufficient quantity of high-quality gametes, as well as ensuring sufficient genetic variability of progeny, which is important for conservation programs [19]. To avoid a shortfall of good-quality sperm, hatcheries usually secure a greater number of males than is minimally needed for reproduction. A viable alternative would be to ensure a more uniform supply of quality sperm by managing the male-to-male sperm variability.

The sterlet has a relatively small body size and one of the shortest reproductive cycles in the family, consequently it has been considered as a model fish for studies in the artificial propagation of sturgeons [20]. The present study tested techniques to maximize the supply of high-quality sperm by (1) incubating spermatozoa with good motility (GS) and bad motility (BS) in seminal plasma collected from these sources, *i.e.* good-quality sperm seminal plasma (GSP) and bad-quality sperm seminal plasma (BSP), with the goal of increasing motility, velocity and fertility parameters of BS, and (2) analyzing GSP and BSP to characterize variation in ionic composition, pH, osmolality and protein concentration.

2. Materials and methods

All experiments were conducted according to the principles of the EU-harmonized Animal Welfare Act of the Czech Republic. Manipulations with animals were performed according to authorization for breeding and delivery of experimental animal reference number: 56665/2016-MZE-17214 (17OZ19180/2016–17214), valid for 5 years from 4 October 2016 issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia by Ministry of Agriculture of the Czech Republic.

2.1. Fish, spermiation and sperm collection

The research was carried out at the experimental station of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Six mature sterlet males and three mature females (males: 12–17 years old, 1–3 kg body weight; females: 12–17 years old, 2–3 kg body weight) were use in the study. Fish manipulation and spermiation followed the techniques as previously described [20]; ovulation was induced with carp pituitary extract dissolved in 0.9% (w/v) NaCl solution administered at 0.5 mg/kg body weight as a priming dose, and a second injection of 4.5 mg/kg body weight was given 12 h later. Eggs were collected into plastic bowls by gentle abdominal massage 30 h after the second injection, then stored under aerobic conditions under a cover (the proportion of eggs was 30% and the proportion of air 70%) at 17 °C in the incubator; these were used at 15 min and 6 h post-collection. Sperm was collected by plastic catheters (4 mm diameter), transferred to plastic boxes 24 h after injection, then stored on ice under aerobic conditions. Sperm motility was checked through a microscope, and sperm samples were separated into two groups: (1) motility > 80% from three males; (2) motility < 20% from three males.

2.2. Incubation of sperm samples for motility and velocity experiment

Sperm samples from the two groups, good motility sperm (GS) and bad motility sperm (BS), were centrifuged individually at 13,000×*g*, 4 °C for 10 min (Thermo Scientific, Fresco 21). The seminal plasma (supernatant) was collected individually as, good seminal plasma (GSP) and bad seminal plasma (BSP). The sperm was reciprocally incubated (identified as GS1-GSP1, GS1-GSP2, etc.; Table 1) with GSP and BSP. A total of 36 reciprocal dilution combinations was formed by combining GS or BS with GSP and BSP in a total of six males. The sperm to seminal plasma dilution ratio (1 : 9 - one part of sperm: nine parts of seminal plasma) produced sample combinations, each of 1 mL; these were placed in 1.5 mL tubes and stored up to 96 h on ice. The GS and BS were individually stored up to 96 h on ice and used as controls (Table 1). Samples of GSP and BSP were used to analyze ion concentration, osmolality, pH and protein concentration.

2.3. Spermatozoon motility and velocity assessment

Motility was initiated by diluting sperm samples at a ratio of 1:40 in an activation medium [10 mM Tris–HCl buffered at pH 8.0 containing 0.25% Pluronic F-127 (to prevent spermatozoon from adhering to microscope slides)] and examined microscopically under 10 × lens, negative phase-contrast condenser with ISAS digital camera (PROISER, Spain) equipped with uEye Cockpit software setting at 25 frame/s after 15 min, 6 h, 24 h and 96 h storage of sperm. Video records were saved in AVI format and analyzed by the Integrated System for Semen Analysis software (PROISER, Spain) at 20 s post-activation. Computer-assisted sperm analysis (CASA) included spermatozoon curvilinear velocity over the actual path (VCL, μm/s), straight-line velocity (VSL, μm/s) and percentage of motile spermatozoa (motility rate, %). Analyses were carried out in triplicate and VCL <10 μm/s was considered as non-motile. All observations were performed at room temperature (21 °C).

2.4. Spermatozoa concentration, seminal plasma osmolality, pH, ion concentration and protein concentration assessment

The spermatozoa concentration was evaluated using a Burker cell hemocytometer (Marienfeld, Germany) with an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) for following fertilization assay. The osmolality of seminal plasma from different males was measured using a Freezing Point OSMOMAT 3000 (Gonotec, Germany) and was expressed in mOsm/kg. The pH of seminal plasma was determined using a laboratory pH meter 320 (WTW, Germany). Concentrations of sodium (Na^+), potassium (K^+) and chloride (Cl^-) ions were measured by potentiometry using Ion Selective Electrodes (ISE, Bayer HealthCare, USA). Calcium (Ca^{2+}) ion concentration was measured by absorption photometry applying the o-cresolphthalein complexone method [21]. Bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, USA) was

Table 1
The reciprocal incubation groups of good-motility sperm (GS) and bad-motility sperm (BS) diluted with seminal plasma from GS (GSP) and seminal plasma from BS (BSP) (dilution 1 : 9) of six individual males (the abbreviation is followed by the male number). The control is similar but undiluted GS or BS.

	GSP1	GSP2	GSP3	BSP1	BSP2	BSP3	Control
GS1	GSP1-GS1	GSP2-GS1	GSP3-GS1	BSP1-GS1	BSP2-GS1	BSP3-GS1	GS1
GS2	GSP1-GS2	GSP2-GS2	GSP3-GS2	BSP1-GS2	BSP2-GS2	BSP3-GS2	GS2
GS3	GSP1-GS3	GSP2-GS3	GSP3-GS3	BSP1-GS3	BSP2-GS3	BSP3-GS3	GS3
BS1	GSP1-BS1	GSP2-BS1	GSP3-BS1	BSP1-BS1	BSP2-BS1	BSP3-BS1	BS1
BS2	GSP1-BS2	GSP2-BS2	GSP3-BS2	BSP1-BS2	BSP2-BS2	BSP3-BS2	BS2
BS3	GSP1-BS3	GSP2-BS3	GSP3-BS3	BSP1-BS3	BSP2-BS3	BSP3-BS3	BS3

used to determine the protein concentration in the samples of seminal plasma and spermatozoa, using the Infinite M200 photometer (Tecan, Männedorf, Switzerland).

2.5. Experiment with fertilization and hatching assay

Equal numbers of eggs from three females were collected and pooled in order to reduce variation in fertilization rates related to egg quality. The BS-BSP and BS-GSP diluted sperm were used together with GS and BS in the fertilization assay (Table 1). Diluted BS-BSP, BS-GSP, GS and BS from individual males were pooled in equal proportions and used as four pooled treatment incubated on ice. Prior to fertilization, the concentration of pooled sperm was determined. Eggs (0.6 g, about 50 eggs) were inseminated with 250,000 spermatozoa (5000 per egg; 2.17 μL of BS and 21.7 μL of BS/BSP, BS/GSP), or 1,250,000 spermatozoa (25,000 per egg; 1.5 μL of GS, 10.9 μL of BS and 108.7 μL of BS/BSP, BS/GSP) by pipetting into 25 mL beakers after 15 min or 6 h of gametes storage, respectively. Only in the case of highly concentrated GS with 250,000 sperm was it impossible to pipette precisely. Therefore, a 1 : 9 dilution with GSP and final volume of 3.09 μL was used. Four beakers for each treatment were placed on a mixing table and 8 mL of dechlorinated water was added into each beaker and mixed for 2 min (150 rpm) at 17 °C. Then, the fertilized eggs from each beaker were gently dispersed into four glass Petri dishes and filled with dechlorinated water. Petri dishes were placed in a small plastic box 1/3 filled with dechlorinated water (300 mL). Incubation was in an air-conditioned laboratory (17 °C). Dechlorinated water in the small plastic boxes was gently changed at 24 h post-fertilization; non-developing embryos (whitish eggs) were removed; the water was exchanged daily after neurulation (72 h post-fertilization) until hatching. Embryo development was recorded by a digital camera (Olympus Corp., Japan, model E-M5MarkII) at 5 h to document the initial total number of eggs, and at 72 h post-fertilization (neurula stage). Viable eggs at the neurula stage were calculated relative to the initial total number of eggs from the photographic records as fertilization rate. Incubation was continued until hatching at 144 h post-activation. Hatched larvae were manually counted directly and compared with the initial number of eggs per Petri dish.

2.6. Statistical analysis

The effect of GSP and BSP on the sperm (good and bad) motility parameters and fertilization success following storage time was analyzed using a multiple ANOVA. The data distribution homogeneity of dispersion was evaluated using Levene's test. The effect of ion concentration, osmolality, pH, protein concentration relative to GSP and BSP was performed by one-way ANOVA. All the differences among means were determined by the LSD test. The results are presented as mean ± SEM. All analyses were performed at a significant level of 0.05 by using R [22].

3. Results

3.1. Effects of GSP and BSP on sperm motility parameters

The quality parameters of sperm (good- and bad-quality spermatozoa, GS and BS) varied significantly ($P < 0.05$) immediately after collection (Figs. 1–3); these included motility rate, curvilinear velocity and straight-line velocity. The parameters changed after incubation of GS and BS in GSP and BSP, respectively. We found that the motility rate of BS increased after incubation with GSP for 15 min, 6 h, 24 h and 96 h (Fig. 1). Similar results were found in curvilinear velocity (Fig. 2) and straight-line velocity (Fig. 3). Moreover, the motility rate of GS after storage in BSP for 15 min, 6 h, 24 h and 96 h decreased compared with GS incubated with or without GSP (Fig. 1). Likewise, the curvilinear velocity (Fig. 2) and straight-line velocity (Fig. 3) of GS after incubation with BSP also decreased with storage time. Multiple analysis showed that storage times and different incubation groups of sperm had significant influence on sperm motility, curvilinear velocity and straight-line velocity.

3.2. Fertilization success of BS after incubation with GSP

The fertilization rate of BS was significantly lower ($P < 0.05$) than GS at 15 min and 6 h (Fig. 4). The fertilization rate of BS significantly increased ($P < 0.05$) after 15 min and 6 h storage in GSP. Furthermore, the fertilization rate of BS after incubation with GSP (71.49 ± 3.71%) at 15 min was not statistically different ($P > 0.05$) from the fertilization level of GS (75.36 ± 3.33%). In addition, the fertilization rate of BS with 1,250,000 spermatozoa was higher ($P > 0.05$) than that with 250,000 spermatozoa after 15 min storage.

Similarly, hatching rate for GS was significantly higher ($P < 0.05$) than BS both at 15 min and 6 h (Fig. 5). The hatching rate of BS increased significantly ($P < 0.05$) after incubation with GSP for both 15 min and 6 h. Additionally, the hatching rate of BS with 1,250,000 spermatozoa was higher ($P > 0.05$) than that with 250,000 spermatozoa after 15 min storage. Multiple analysis showed that both of storage time and quantity of sperm had a significant influence on fertilization and hatching rate.

3.3. Variation of osmolality, pH, ion concentration and protein concentration between GSP and BSP

The Na^+, K^+, Cl^- and osmolality of GSP were significantly higher ($P < 0.05$) than in BSP (Table 2). However, Ca^{2+}, protein concentration and pH were not significantly different between GSP and BSP.

4. Discussion

In the present study, the motility of sterlet sperm varied significantly after collection under hatchery conditions. Interestingly, the motility rate, VCL, VSL and fertilizing capacity of BS were

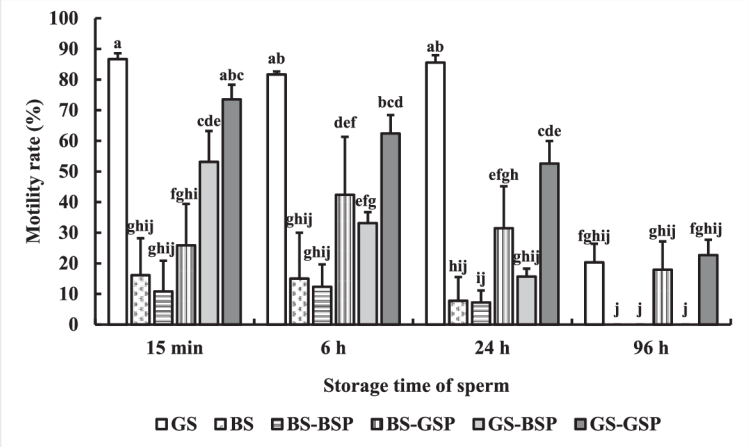

Fig. 1. Motility rate (%) of different groups of sterlet *Acipenser ruthenus* sperm at 20 s post activation after storage of 15 min, 6 h, 24 h and 96 h. Data are expressed as mean +SEM. Values with a different lowercase letter are significantly different ($P < 0.05$). GS: sperm with good motility; BS: sperm with bad motility; BS-BSP: bad-motility sperm incubated with seminal plasma from bad-motility sperm; BS-GSP: bad-motility sperm incubated with seminal plasma from good-motility sperm; GS-BSP: good-motility sperm incubated with seminal plasma from bad-motility sperm; GS-GSP: good-motility sperm incubated with seminal plasma from good-motility sperm.

found to be increased significantly after storage in GSP. Low quality sperm can result from males at different maturity levels [2]. We know that immature sterlet testicular spermatozoa are immotile but can attain activation potential and become mature after incubation in urine or seminal plasma from the Wolffian duct [16]. This is somewhat similar to the revitalization of low-quality sterlet sperm by storage in GSP demonstrated here.

In the current study, storage of sperm in GSP or BSP for 15 min was sufficient to improve sperm motility. A previous study on carp sperm demonstrated restored ATP and reinitiated motility after treatment [23]. The motility re-initiation was achieved by incubating sperm for several minutes in an isotonic solution, which allowed re-loading of energy in spermatozoa. Similarly, artificial seminal plasma had a beneficial effect on common carp sperm motility by rebuilding the energy reserves necessary for movement [18]. In the present study, storage of sperm to acquire motility might also result from the reloading of ATP. Further, prolonging storage time of BS in GSP to 6 or 24 h did not significantly improve their motility or velocity, although a decrease in some motility parameters were observed after 96 h. This finding is important for practical applications, *i.e.* collect the seminal plasma from other good quality sperm in subsequent stripping, then incubate low quality sperm for several hours before another egg collection.

We observed that an increase in sperm motility and velocity improved their capacity for fertilization; GSP sterlet sperm had a beneficial effect on sperm motility by probably regenerating energy. The fertilization and hatching rates of BS storage in GSP after 6 h with 250,000 spermatozoa (32.89 ± 3.41% and 15.41 ± 2.56%) was significantly decreased compared to storage for 15 min (64.77 ± 6.03% and 55.12 ± 6.72%). This may be due to the decrease of egg quality after 6 h storage, although it was shown that no significant decrease of sterlet egg quality after 6 h of storage at 15 °C or 19 °C [24]. Increase of fertilization and hatching rates of BS stored in GSP after 6 h with 1,250,000 spermatozoa compared with

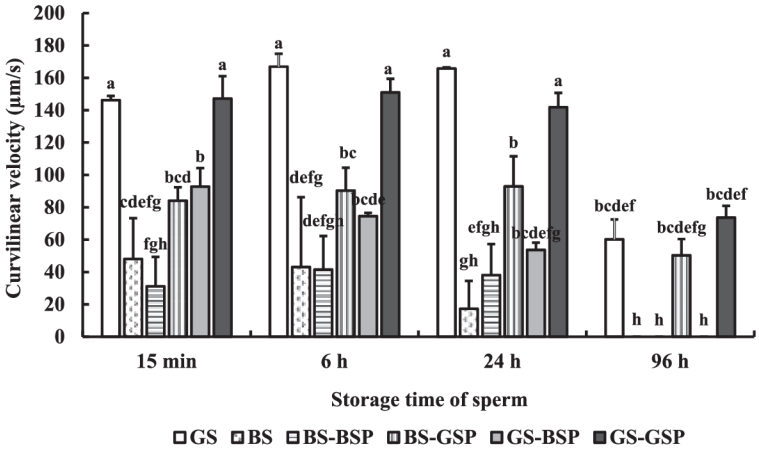

Fig. 2. Curvilinear velocity (μm/s) of different groups of sterlet *Acipenser ruthenus* sperm at 20 s post activation after storage of 15 min, 6 h, 24 h and 96 h. Data are expressed as mean +SEM. Values with a different lowercase letter are significantly different ($P < 0.05$). GS: sperm with good motility; BS: sperm with bad motility; BS-BSP: bad-motility sperm incubated with seminal plasma from bad-motility sperm; BS-GSP: bad-motility sperm incubated with seminal plasma from good-motility sperm; GS-BSP: good-motility sperm incubated with seminal plasma from bad-motility sperm; GS-GSP: good-motility sperm incubated with seminal plasma from good-motility sperm.

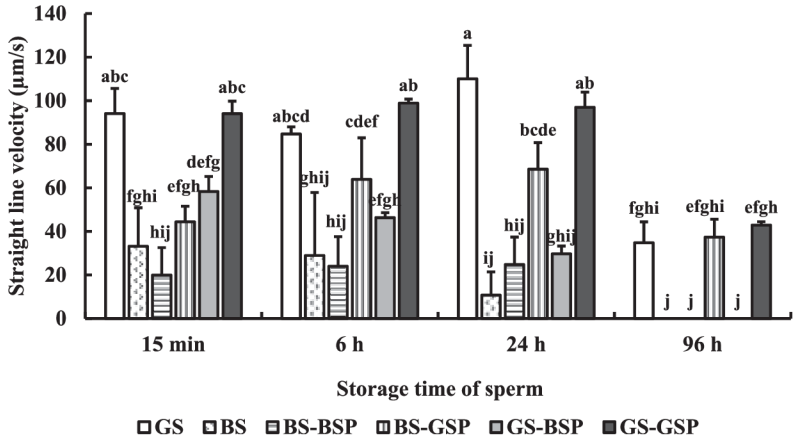

Fig. 3. Straight line velocity (μm/s) of different groups of sterlet *Acipenser ruthenus* sperm at 20 s post activation after storage of 15 min, 6 h, 24 h and 96 h. Data are expressed as mean +SEM. Values with a different lowercase letter are significantly different ($P < 0.05$). GS: sperm with good motility; BS: sperm with bad motility; BS-BSP: bad-motility sperm incubated with seminal plasma from bad-motility sperm; BS-GSP: bad-motility sperm incubated with seminal plasma from good-motility sperm; GS-BSP: good-motility sperm incubated with seminal plasma from bad-motility sperm; GS-GSP: good-motility sperm incubated with seminal plasma from good-motility sperm.

250,000 spermatozoa illustrated that a higher number of spermatozoa could increase fertilization success, which may be a result of the variation of total motile sperm. The use of a total of 250,000 spermatozoa, however, had fewer motile spermatozoa after 6 h storage, which may lead to the decreased fertilization rates, while an increase of total number of spermatozoa up to 1,250,000, resulted in a greater number of actual motile spermatozoa. This may be because increasing the number of spermatozoa, compensates for the low motility rate [25]. From a practical point of view, we suggest that higher spermatozoon quality can be achieved by storing for 15 min in GSP. The use of sperm of good quality reduces the risk of obtaining a low fertilization rate. Improvement of sperm quality with low motility originally by storage could ensure higher success of fertilization and maintain higher genetic diversity, especially for endangered species, when there is usually a limited number of broodstock and gametes during each striping.

Since the GSP can improve the quality of BS, and BSP decreased the quality of GS during storage, we considered how the inorganic composition characteristics of GSP and BSP differed. We found that the Na^+, K^+ and Cl^- ions and osmolality were significantly higher in GSP than in BSP. In sturgeon species, the Na^+, K^+ and Cl^- ions are the main inorganic components in seminal plasma and play a crucial role in maintaining osmotic balance during sperm storage [6]. Thus, the significantly higher concentrations of these ions in GSP may be one of the factors in osmolality differences. Osmolality change is a primary factor that triggers spermatozoa activation or inhibition. Sperm are quiescent in the high osmolality of seminal plasma and are activated when exposed to low osmolality of the external environment. Sturgeons and paddlefishes usually have low osmolality (30–60 mOsmol/kg) due to the dilution of sperm by low osmolality urine (32 ± 8 mOsmol/kg) from the kidney and the seminal plasma maintains the sperm immotility [6,16,26,27]. In contrast, seminal plasma of teleosts is not mixed with urine, thus maintaining a higher osmolality and sperm immotility; osmolality in cyprinids is 300–400 mOsmol/kg and salmonids is 200–350 mOsmol/kg [6]. In the present study, the osmolality of GSP in sterlet was 31.66 ± 1.33 mOsmol/kg while the osmolality was lower in BSP at 14.66 ± 1.20 mOsmol/kg, thus suggesting one of the causes for

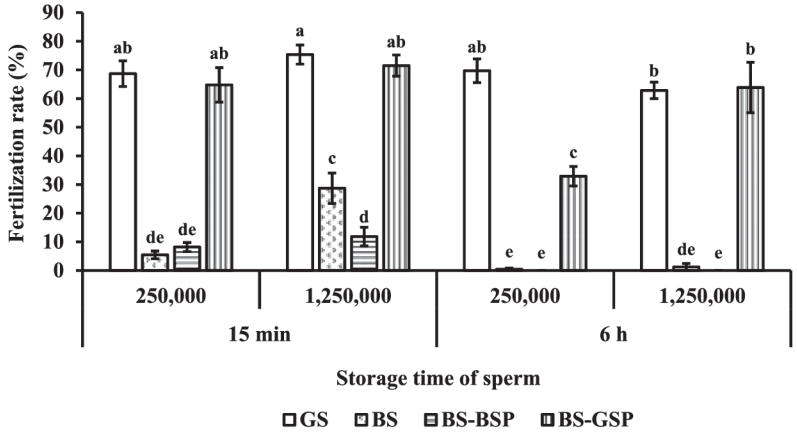

Fig. 4. Fertilization rate (%) of different groups of sterlet *Acipenser ruthenus* sperm after storage of 15 min and 6 h with total of 250,000 and 1,250,000 spermatozoa, respectively. Data are expressed as mean +SEM. Values with a different lowercase letter are significantly different ($p < 0.05$). GS: sperm with good motility; BS: sperm with bad motility; BS-BSP: bad-motility sperm incubated with seminal plasma from bad-motility sperm; BS-GSP: bad-motility sperm incubated with seminal plasma from good-motility sperm.

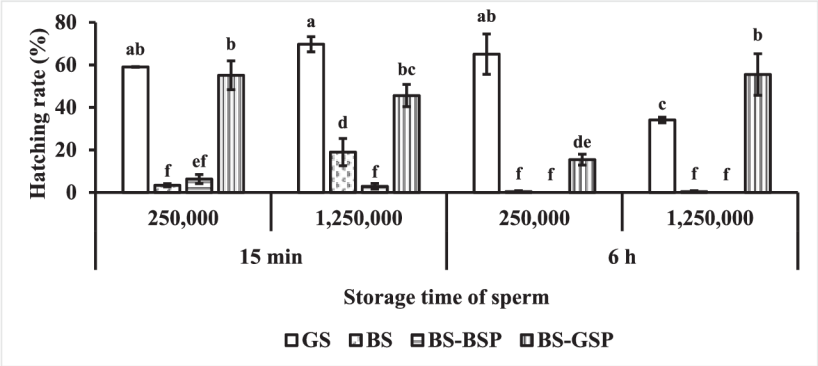

Fig. 5. Hatching rate (%) of different groups of sterlet *Acipenser ruthenus* sperm after storage of 15 min and 6 h with total of 250,000 and 1,250,000 spermatozoa, respectively. Data are expressed as mean +SEM. Values with a different lowercase letter are significantly different ($P < 0.05$). GS: sperm with good motility; BS: sperm with bad motility; BS-BSP: bad-motility sperm incubated with seminal plasma from bad-motility sperm; BS-GSP: bad-motility sperm incubated with seminal plasma from good-motility sperm.

Table 2
Chemical characteristics (mean ± SEM) of seminal plasma from good- (GSP) and bad- (BSP) motility sperm of sterlet.

	$[Na^+]$ (mM)	$[K^+]$ (mM)	$[Ca2^+]$ (mM)	$[Cl^-]$ (mM)	Osmolality (mOsmol/kg)	Protein concentration (μg/mL)	pH
GSP	16.70 ± 0.59*	2.48 ± 0.24*	0.10 ± 0.014	5.00 ± 0.25*	31.66 ± 1.33*	147.98 ± 54.73	8.13 ± 0.16
BSP	5.23 ± 0.86*	1.20 ± 0.30*	0.185 ± 0.08	3.33 ± 0.49*	14.66 ± 1.20*	95.39 ± 37.21	7.88 ± 0.25

poorer sperm quality.

Moreover, ions such as K^+ and osmolality are essential factors in sperm motility inhibition in sturgeons [27,28]. The presence of K^+ ion in the external environment at an appropriate concentration inhibits spermatozoa motility [27]. The initiation of sperm motility of the Mississippi paddlefish *Polyodon spathula*, Persian sturgeon *Acipenser persicus* and the lake sturgeon *Acipenser fulvescens* is triggered by a reduction of K^+ concentration or is attenuated by an increase of K^+ concentration in the external environment [28–31]. Flux of the K^+ ions through the plasma membrane plays a key role in the maintenance of membrane potential. A decrease in the external K^+ concentration induces K^+ efflux from the cell and by membrane hyperpolarization [32,33]. Membrane hyperpolarization triggers a cascade of events leading to spermatozoa activation through cyclic AMP-dependent phosphorylation of axonemal proteins or Ca^{2+}-dependent calmodulin phosphorylation [34,35]. It has been demonstrated that 0.5 mM K^+ can fully inhibit sperm movement in paddlefishes [27]. In the present study, the concentration of K^+ in BSP (1.2 ± 0.3 mM) was higher than 0.5 mM, which should be sufficient to prevent sterlet sperm movement. Furthermore, we observed that sperm are kept immotile in BSP, but the motility parameters of GS decreased dramatically after incubation in BSP (Figs. 1–3). This suggested that K^+ inhibits sperm motility but is not the only factor in maintaining sterlet sperm quality during storage. Also, inhibition of sperm motility is not the only factor which maintains sperm quality during storage. Probably K^+, osmolality and other ions contribute to the storage of sterlet sperm. It has been demonstrated that the presence of some ions can overcome the inhibitory effect of the K^+ ions, *e.g.* Ca^{2+} and Na^+ ions [33,36,37]. It has been reported that 0.25 mM of $CaCl_2$ reinitiated paddlefish sperm motility when blocked by 0.5 mM KCl [27]. In the present study, the concentration of Ca^{2+} in BSP was 0.185 ± 0.08 mM, which may be insufficient for reactivation of sterlet sperm (K^+ in BSP; 1.20 ± 0.30 mM).

Some organic components, such as enzymes and other proteins also play essential roles to protect sperm from damage during storage [38]. Testicular sterlet sperm can mature and acquire motility after incubation with seminal plasma but result in a lack of motility after incubation with seminal plasma with removal of high molecular weight substances (>10 kDa) [16]. Some seminal plasma proteins may be related to the final maturation process of spermatozoa and nutrients supply for sperm metabolism [11,16]. Thus, we propose that proteins in sterlet GSP might also play a role in improving sperm quality and protecting sperm during storage, especially when the low sperm quality is caused by immaturity. However, the total concentration of proteins in GSP and BSP showed no significant difference in the current study.

5. Conclusion

In conclusion, the revitalization of sterlet sperm is possible in a short time using natural GSP. The application of GSP improves sperm motility and velocity during 15 min, 6 h, 24 h and 96 h, regardless of the low quality of the fresh sperm collected. We also demonstrated that the fertilization capacity of the revived sperm reached similarly high level of good quality fresh sperm with just 5000 spermatozoa per egg. Therefore, the storage of bad-motility sperm with GSP for a short time is suggested to increase the fertilization rate in hatcheries. Moreover, factors in seminal plasma which determine sperm quality include osmolality, K^+ and Na^+ by protecting sterlet sperm during storage. Further analysis of antioxidants and proteome in GSP and BSP is needed to precisely understand all of the factors involved in the relationship of GSP and BSP to sperm motility. Additionally, experiments with GSP and artificial immobilizing solution with or without proteins or lipids to improve the quality of low motility sperm should also be performed.

CRediT authorship contribution statement

Miaomiao Xin: Conceptualization, Investigation, Resources, Data curation, Methodology, Writing - original draft, Writing - review & editing, Validation, Supervision. **Yu Cheng:** Conceptualization, Investigation, Resources, Data curation, Methodology, Writing

- original draft. **Marek Rodina:** Resources, Investigation, Project administration. **Vladimíra Tučková:** Resources, Investigation, Project administration. **William L. Shelton:** Writing - review & editing, Validation, Supervision. **Otomar Linhart:** Conceptualization, Investigation, Resources, Data curation, Methodology, Writing - original draft, Writing - review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no competing interests.

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CHAPTER 5

PRACTICAL USE OF EXTENDER AND ACTIVATION SOLUTIONS FOR SHORT-TERM STORAGE OF COMMON CARP (*Cyprinus carpio* L.) MILT IN A HATCHERY

Cheng, Y.#, Zhang S.P.#, Linhartová Z., Shazada N.E., Linhart O., 2022. Practical use of extender and activation solutions for short-term storage of common carp (*Cyprinus carpio*) milt in a hatchery. Aquaculture Reports 24, 101160.

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Practical use of extender and activation solutions for short-term storage of common carp (*Cyprinus carpio*) milt in a hatchery

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ABSTRACT

The purpose of this study was to investigate the effect of an activation solution (AS) (hatchery water (pH 7.9 and 4 mOsm/kg), Perchec solution (5 mM KCl, 45 mM NaCl, 30 mM Tris-HCl; pH 8.0 and 130 mOsm/kg) and Cheng solution (40 mM NaCl, 20 mM Tris; pH 8.0 and 100 mOsm/kg) on aging sperm of common carp (*Cyprinus carpio*) after storage in a carp artificial seminal plasma (carp ASP: 2 mM $CaCl_2$, 1 mM $MgSO_4$, 40 mM KCl, 110 mM NaCl, 20 mM Tris; pH 7.5 and 310 mOsm/kg) for 7 days without antibiotics at 4 °C. Fresh milt and undiluted aging milt were used as the control groups. Milt (5 ml) was collected and diluted 1:1 in a carp ASP in 50 ml containers and stored at 4 °C under aerobic conditions. The highest sperm motility was found 45% and 36% at 15 s and 45 s post sperm activation (PSA) in hatchery water. Additionally, the curvilinear velocity (VCL, μm/s) and straight-line velocity (VSL, μm/s) of sperm were the highest in hatchery water and the solution with 5 mM KCl, 45 mM NaCl, 30 mM Tris and pH 8.0, which were above 122 μm/s and 87 μm/s at 15 PSA. Sperm concentration in aging sperm of common carp is known to decrease rapidly, so prior to fertilization, sperm concentration was determined and accurately set to 200,000 sperm per egg. The fertilization of eggs and hatching rate after 7 days of sperm storage in carp ASP were 85–87% and 78–81% in both ASs of hatchery water and Perchec solution. Less than 1% sperm motility, fertilization and hatching rate were observed in undiluted sperm (control). In conclusion, the fertilization ability of aging common carp sperm in carp ASP within different ASs was confirmed for practical use in aquaculture.

1. Introduction

Short-term storage and management of milt is a very crucial part in hatcheries. It is a simple and economical process that is frequently used with the following beneficials: to provide a steady supply of fry by maintaining a continuous supply of milt; to reduce the requirement of maintaining broodstock available for a long time; to induce *in vitro* maturation of sex-reversed males' sperm; to transport milt from fish farming to other locations for egg fertilization; to keep milt available when sexual maturity is asynchronous and so on (Contreras et al., 2020). Usually, the factors that can influence storage of fish milt are considered. For example, the quality of the broodstock (*i.e.* age, breeding season, diet, health and management), fresh milt quality and quantity (*i.e.* volume, sperm concentration and motility), and the conditions of milt storage (*i.e.*, temperature, artificial seminal plasma, time, volume, perturbations and atmosphere) (Contreras et al., 2020). After considerable research dealing with fish milt storage, great progress has been achieved. New techniques, for example proteomics and epigenetics during sperm storage (Cheng et al., 2021; Dietrich et al., 2021), on aging sperm in common carp (*Cyprinus carpio*) have been developed, which provides a crucial tool for the future development of novel potential molecular markers of sperm quality and helped us to improve the sperm preservation protocols in aquaculture. However, in practice, dynamic changes of sperm concentration, spermatozoa activity and reaction with eggs within specific water environment should be considered in using aging sperm.

With the purpose of prolonging storage time and avoiding deterioration of sperm function, artificial seminal plasma (ASP) has usually

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been applied. A considerable number of research has been explored regarding the ASP in common carp. From these studies, a carp ASP (2 mM $CaCl_2$, 1 mM $MgSO_4$, 40 mM KCl, 100 mM NaCl, 20 mM Tris; pH 7.5 and 310 mOsm/kg) (Cejko et al., 2018) has recently been confirmed as one of the most valuable ASPs in common carp sperm (Cejko et al., 2022; Cheng et al., unpublished data). After 14 days of sperm storage in a carp ASP with a large milt volume and antibiotics, the fertilization of eggs and the rate of hatching did not differ significantly from the fertilization and hatching rate when fresh sperm was used. Storage in carp ASP can also keep more spermatozoa than milt stored without dilution in carp ASP and other ASPs after 8 days at 4 °C (Cheng et al., unpublished data). The carp ASP largely enhances the aging sperm quality in common carp (compared to that without dilution), which enables milt to be stored during the short-term if needed in the hatchery. However, there are limited data to support the role of different AS on aging sperm, stored in an effective ASP, and subsequently used for fertilization.

Until now, limited attention has been paid to the effect of ASs on the functions and fertilization capacity of aging sperm after short-term storage. Most of the existing studies have focused on sperm activation and fertilization capacity of fresh sperm using various ASs. Osmolality and ionic composition are the vital properties of ASs in sperm motility and fertilization success (Perchec et al., 1995; Alavi and Cosson, 2006). An AS with an osmolality of 100–150 mOsm/kg has been found to be most successful in common carp (Saad and Billard, 1987; Billard et al., 1995). In a further study Żarski et al. (2015) confirmed that an AS with an osmolality of 100 mOsm/kg was the most suitable for common carp egg fertilization. Meanwhile, the significant effect of the AS ionic composition, with a combination of Na^+/K^+ and extracellular Ca^{2+}, on the sperm activation and fertilization success of fishes has also been reported (Saad and Billard, 1987; Linhart et al., 1999; Alavi and Cosson, 2006). Further, the motility of spermatozoa in freshwater fishes is influenced by the pH of the activation medium; alkaline conditions are preferable for the initiation of sperm activation in freshwater fishes (Krasznai et al., 2003). However, for some species such as rainbow trout (*Oncorhynchus mykiss*), this fact has not been confirmed specifically, when it comes to sperm after short-term storage *in vitro* (Nynca et al., 2012).

Fertilization success in fishes is associated with the type of movement, trajectory and duration time of the sperm, which are all influenced by the AS (Alavi and Cosson, 2005). Teleost spermatozoa usually move in a straight, slightly curved path, but at the surface or bottom in a volume of AS they have a different type and direction of movement (Boryshpolets et al., 2013). Under conditions such as end of motion, presence of toxins, inappropriate formulation of the dilution medium or higher content of Ca^{2+}, the movement becomes more curved and even circular (Cosson et al., 1999; Rurangwa et al., 2004). Moreover, the AS may affect the motility duration (Cosson et al., 1999; Alavi et al., 2019; Nahiduzzaman et al., 2014). The time to activate eggs and the duration of egg fertilization ability is also affected by the corresponding AS (Linhart et al., 2004; Zarski et al., 2012). However, the spermatozoa must adhere to and enter the micropyle of the egg during a limited period, which is usually shorter for sperm movement than for the micropyle closure time (Ginsburg, 1972). Therefore, it is one of the key factors that spermatozoa activated by a suitable AS which enhances movement ability and longer duration time for the successful fertilization.

The aim of the study was to determine a practical method of common carp sperm quantity adjustment prior to fertilization and fertilization ability measurement on an industrial scale with different ASs on aging sperm after storage in carp ASP under aerobic conditions at 4°C for 7 days.

2. Material and methods

2.1. Ethics statement and animals

The study was carried out at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic. Manipulations with animals were performed according to authorization for breeding and delivery of experimental animals (Reference number: 56665/2016-MZE-17214 and 64155/2020-MZE-18134) and permission to use experimental animals (Reference number: 68763/2020-MZE-18134) issued to the Faculty of Fisheries and Protection of Waters, the University of South Bohemia by the Ministry of Agriculture of the Czech Republic. There were no endangered or protected species involved in the study. Authors of the study (ZL, OL) own the Certificate of professional competence for designing experiments and experimental projects under Section 15d (3) of Act no. 246/1992 Coll. on the Protection of Animals against Cruelty.

2.2. Fish, spermiation, ovulation, and milt and egg collection

Twelve mature common carp males (six for milt storage and six for fresh sperm, 3 years old, 1.5–2.5 kg body weight) and five females (6 years old, 7.5–8.5 kg body weight) were used in this study. Fish manipulation and spermiation followed the techniques previously described (Linhart et al., 2015). Before hormonal application and gametes collection, the males and females were anaesthetized in a solution of 2-phenoxyethanol (1:1000, Sigma-Aldrich, St. Louis, MO, USA). A single intramuscular injection of carp pituitary extract (CP) was administered to each of the nine males at a dose of 2 mg/kg body weight with 0.9% (*w/v*) NaCl solution. A syringe was used to collect milt 24 h after hormonal application from each male during gentle abdominal massage, then milt was stored on ice under aerobic conditions according to the experimental design. Five females, in good physical condition, were selected. After a 3-day period of acclimation in the hatchery at 20 °C, ovulation of the females was induced using CP dissolved in 0.9% (w/v) NaCl solution; 0.5 mg/kg body weight was treated as a priming dose and a second dose of 2.7 mg/kg of body weight was administered 12 h later. Eggs were collected into plastic bowls 12 h after the second hormonal treatment. The best quality eggs (darker coloration, without excessive ovarian fluid and no apparent decomposition) were visually selected from five individual females and pooled eggs were immediately used for the fertilization assay.

2.3. Milt storage

The quality of fresh sperm was checked with the computer-assisted sperm analysis (CASA) system. Milt with good quality (motility > 90%) was collected from six males, and then the samples were divided into two portions. One portion of 10 ml milt was stored without dilution (control) in a 50 ml container for cell culture made of polystyrene designed for aerobic conditions (Thermo Scientific Nunc). The other portion of 5 ml milt was diluted 1:1 with 5 ml carp ASP containing 2 mM $CaCl_2$, 1 mM $MgSO_4$, 40 mM KCl, 110 mM NaCl, 20 mM Tris at pH 7.5 and 310 mOsm/kg (Cejko et al., 2019). Milt without and with dilution in carp ASP from each male was preserved on ice separately for 7 days without antibiotics.

2.4. Spermatozoa motility, velocity and concentration evaluation during short-term storage of milt in vitro

Sperm samples of undiluted (control, $n = 6$) and diluted in carp artificial seminal plasma (ASP, $n = 6$) after 7 days of sperm storage (DSS), and also fresh sperm ($n = 3$) before fertilization were analyzed by the CASA system. The ASs including hatchery water (pH 7.9 and osmolality 4 mOsm/kg), Perchec solution (5 mM KCl, 45 mM NaCl, 30 mM Tris-HCl, pH 8, osmolality 130 mOsm/kg) (Perchec et al., 1996) and

Cheng solution (one new solution that used in this study composed of 40 mM NaCl plus 20 mM Tris, pH 8.0, osmolality 100 mOsm/kg), were used to trigger sperm motility during the analysis. To prevent adherence of the sperm samples to the microscope slide, 0.25% bovine serum albumin (BSA; Sigma-Aldrich; St. Louis, MO, USA) was added to the ASs. Spermatozoa were activated at room temperature (21 °C) by mixing the diluted sperm sample with needles into 10 μl of the AS to make sure 100 cells per view on a chamber SpermTrack-10® (Proiser R + D, S.L.; Paterna, Spain). The activated spermatozoa were recorded microscopically till the end of motility and followed the techniques described previously (Cheng et al., 2021). In detailed, microscope (UB 200i, PROISER, Spain) at 10× using a phase-contrast condenser and an ISAS digital camera (PROISER, Spain) equipped with uEye Cockpit software setting at 25 frames s^{-1} were used. Recordings were stored on a hard disk in AVI format. Analyses of the sperm recordings were performed by the Integrated System for Semen Analysis software (PROISER, Spain). Computer-assisted sperm analysis (CASA) included. The CASA parameters included the percentage of motile sperm (%), curvilinear velocity (VCL, μm/s) and straight-line velocity (VSL, μm/s) at 15 s and 45 s of post sperm activation (PSA). Scales in sperm analysis software were calibrated and the base on the microscope and adapter set. Escala X and Escala Y were both set up to 1.38 μm when using 10× lens on a negative phase-contrast condenser microscope. Quantitative analyses of all samples were conducted in triplicate.

Sperm concentration (expressed as 10^9 spz/ml) was evaluated by a Bürker cell hemocytometer (Marienfeld, Germany, 12 squares counted for each male), using an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) under an Olympus microscope BX 41 (4009). For counting the cell number clearly, the Bürker cell hemocytometer containing sperm samples was placed horizontally for about 3 min for the cells to sediment.

2.5. Fertilization, hatching and malformation rate of 7 DSS diluted in carp ASP with AS

To determine the fertilization capacity and quality of eggs, fresh sperm (motility > 90%) from six males was used during the experiment. For reproduction, eggs from five females were pooled. Prior to fertilization, the 7 DSS without dilution (control), 7 DSS with dilution in carp ASP from six males and three parts of fresh sperm without dilution from each two males were pooled, respectively. The sperm concentration (Fig. 1) in pooled fresh sperm and sperm with or without dilution stored in carp ASP for 7 DSS was calculated using a Bürker cell hemocytometer (Marienfeld, Germany, 12 squares counted for each male) with an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) under an Olympus microscope BX 41 (4009). Next, the appropriate volume of sperm as follows: fresh sperm = 2 ml, 7 DSS without dilution in carp ASP (control) = 2.8 ml, 7 DSS diluted in carp ASP = 4.4 ml, was added to 200 g (about 160,000) eggs to obtain a concentration of 200,000 sperm per egg in all fertilization batches. As an activator in the fertilization trial, the ASs that are usually used, hatchery water and Perchec solution, were chosen. Another new AS-Cheng solution was selected from five ASs (10/20/30/40/50 mM NaCl, respectively, with 20 mM Tris, pH 8.0) (see supplementary materials). One hundred ml of AS including hatchery water and Perchec solution Cheng solution were used respectively for each fertilization sample.

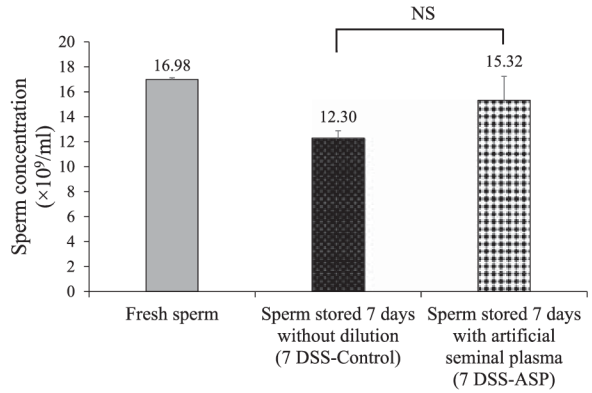

Fig. 1. Sperm concentration of common carp (*Cyprinus carpio*) before fertilization without a carp artificial seminal plasma (ASP; control) and with carp ASP (Cejko et al., 2019) of 7 days sperm storage (DSS) in both cases from six males. Fresh sperm collected at 0 h and used as three parts of pool of sperm from each two males. Sperm concentration multiplied by corresponding dilution factor, × 1 in undiluted group (Fresh sperm and 7 DSS-Control), × 2 in 1:1 diluted in carp artificial seminal plasma group (7 DSS-carp ASP) for convenient comparison. The results are expressed as means ± S.D. NS indicate no significant differences between groups ($P < 0.05$).

Milt was added directly to the eggs in bowls (diameter 15 cm). All bowls were placed on a shaking table (21 °C). The shaking table was activated for 2 min (150 rpm), and 100 ml of AS was added into each bowl during this period.

An unsticking procedure was applied to the fertilized eggs prior to incubation in regular hatchery Weiss jars (10 l) (Fig. 2). The procedure involved 10 min on a shaking table (21 °C) in a bath of tannic acid solution with concentrations ranging between 0.4 and 1.5 g/l, following fertilization. Subsequently after the unsticking procedure, 400 fertilized eggs, were collected, immediately after placing them in the Weiss jars, and gently distributed into four Petri dishes (9 cm in diameter and 1.5 cm in depth), for the fertilization and hatching assays. Each group of four Petri dishes was placed in four plastic boxes (13.5 cm × 10 cm × 6.5 cm) and filled with dechlorinated water (300 ml) (Cheng et al., 2020). The room temperature was controlled at 23.5 °C by air-conditioning. If more than three eggs adhered together, after fertilization and distribution of eggs into the Petri dishes, the cluster was removed using a needle and plastic pipette to avoid future embryonic death accompanied by the development of fungi. Then the total remaining number of eggs was counted and considered as the original total number of eggs. At 48 h post-fertilization, non-developing embryos were removed, and eye-stage embryos (fertilized ones) were counted after taking pictures with use of mobile phones during water exchange. After the eye-stage, dechlorinated water was changed completely and daily up to hatching. Until hatching, the normal and malformed larvae were manually counted by visually over the next 4 days of incubation at 23.5 °C. Fertilization and total hatching rates were calculated as the ratio of all remaining eye-stage embryos at 48 h post-fertilization and hatched larvae at 4 days post-fertilization from the initial number of eggs per Petri dish.

Malformations for larvae were observed under a digital Olympus microscope BX 41 (4009). All larvae with unusual body proportions, and irregular body axis and head, were considered as malformed larvae (Lugowska, 2018). The malformation rate was calculated from total hatched larvae in each Petri dish.

2.6. Statistical analysis

Sperm motility parameters among fresh sperm, 7 DSS in carp ASP and 7 DSS undiluted sperm (control) samples under three ASs [hatchery water, Perchec solution and Cheng solution at 15 s and 45 s PSA were verified using two-way ANOVA respectively. The data distribution homogeneity of dispersion was evaluated using Levene's test. Differences between the sperm concentrations of 7 DSS-Control and 7 DSS- carp ASP were verified using *t*-tests. Fertilization, hatching and malformation rates among the three sperm groups and three ASs were performed by two-way ANOVA. Differences among means between groups were determined with an LSD test for *post hoc* multiple comparisons. Differences in the motility, VCL and VSL at 15 s and 45 s PSA were determined using *t*-tests.

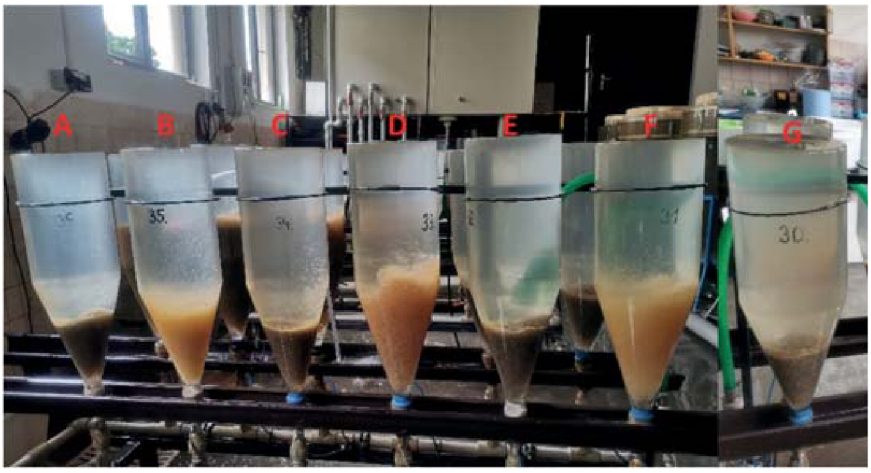

Fig. 2. Hatchery Weiss jars of 10 l used for common carp (*Cyprinus carpio*) egg incubation and hatching after sperm short-term storage for 7 days (the diagram indicates the eye-stage 3 days after fertilization). (A) Fresh sperm activated with hatchery water; (B) 7 days sperm storage (DSS)-control without carp artificial seminal plasma (ASP) activated by hatchery water; (C) 7 DSS-carp ASP activated by hatchery water; (D) 7 DSS-control without ASP activated by Perchec solution; (E) 7 DSS-carp ASP activated by Perchec solution; (F) 7 DSS-control without carp ASP activated by Cheng solution; (G) 7 DSS-carp ASP activated by Cheng solution. Macroscopically, the breakdown of unfertilized eggs in controls (orange-yellow color) is visible, contrasting with dark embryos in incubation bottles where sperm diluted with, without carp ASP or fresh sperm was used.

Bar charts of sperm motility, VCL, VSL, sperm concentration, fertilization, hatching and malformation rates were drawn with mean ± S.D. All analyses from ANOVA were performed at a significant level of $P < 0.05$ in R 3.3.2 (R Core Team, 2019).

3. Results

3.1. Effect of ASP on sperm concentration

Sperm concentration reduced by 3.03 × 10^9/ml in 7 DSS without dilution compared to the 7 DSS where sperm diluted in carp ASP after multiplied by corresponding dilution factor (*i.e.* ×2). (See in Fig. 1).

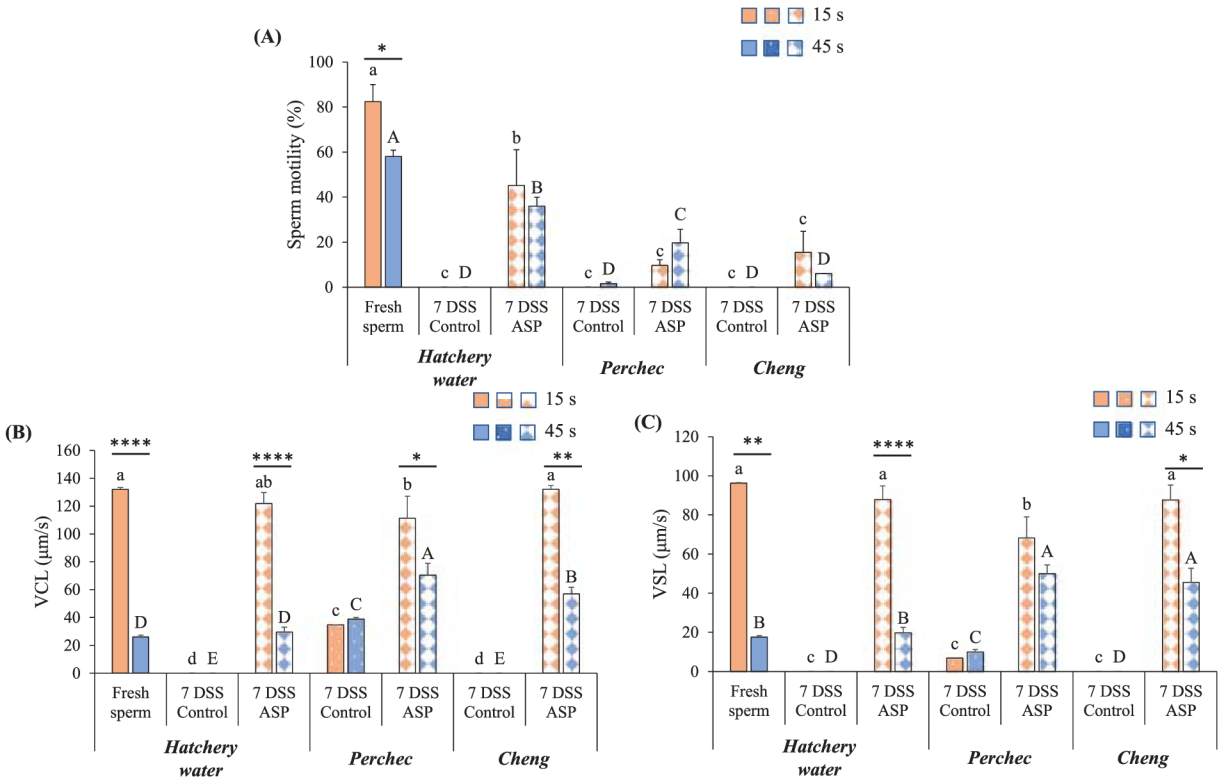

Fig. 3. . (A) Motility rate (%), (B) curvilinear velocity (VCL; μm/s) and (C) straight-line velocity (VSL; μm/s) of common carp (*Cyprinus carpio*) spermatozoa at 15 s and 45 s of post sperm activation (PSA) in the following activation solutions: hatchery water, Perchec solution and Cheng solution. Sperm was stored without carp artificial seminal plasma (ASP; control) and with carp ASP of 7 DSS in both cases from six males. Fresh sperm was collected at 0 h and used as three parts of pool of milt from each two males. Different lowercase letters indicate significant differences among 15 s PSA, different uppercase letters indicate significant differences among 45 s PSA ($P < 0.05$). Results from *t*-tests are presented as the mean ± SD (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

3.2. Effect of activation solutions on CASA parameters after 7 DSS of diluted sperm

In general, after 7 days of sperm short-term storage *in vitro*, motility in diluted sperm with carp ASP was significantly higher than the control which were almost no motile spermatozoa at 15 s and 45 s PSA (Fig. 3A). On the other hand, sperm motility after 7 DSS was significantly lower than fresh sperm. Additionally, hatchery water had the ability to activate more spermatozoa (45%) in carp ASP groups than other ASs (< 15%) whenever in 15 s and 45 s. Moreover, Perchec solution activated more spermatozoa after 45 s PSA rather than 15 s PSA although the obtained result was not significant (Fig. 3A). Motility of sperm at 15 s PSA was higher than 45 s PSA in fresh sperm.

At 15 s PSA, significant differences between undiluted (Control) and diluted (carp ASP) sperm were observed after 7 days for VCL (0–35 μm/s and 110–130 μm/s, respectively, for Control and carp ASP); the highest VCL (> 120 μm/s) were obtained among 7 DSS-carp ASP with hatchery water and Cheng solution, which were similar as fresh sperm activated by hatchery water. However, after sperm was activated at 45 s, Perchec solution achieved the highest VCL in 7 DSS-carp ASP compared to other ASs with aged sperm groups and also similar in fresh sperm group as well (Fig. 3B). On the other hand, VCL in all groups (aged sperm diluted with ASP and fresh sperm) at 15 s PSA were higher than these at 45 s PSA. No differences observed in VCL between 15 s PSA and 45 s PSA among groups without carp ASP during 7 days of short-term storage of sperm.

Similar trend was observed in VSL that carp ASP improved 7 days aged sperm compared to control whenever at 15 s or 45 s PSA. At 15 s PSA, sperm VSL had the similar level (>85 μm/s) in 7 DSS-ASP activated by hatchery water, Cheng solution and fresh sperm, which was differed significantly from the 60 μm/s values determined in 7 DSS-carp ASP with Perchec solution (Fig. 3C). Yet, 7 DSS-carp ASP with Perchec and Cheng solutions achieved highest VSL at 45 s PSA. No changes were found in VSL between 15 s and 45 s PSA, apart from fresh sperm, 7 days-aged sperm in hatchery water and Cheng solution (Fig. 3C).

3.3. Fertilization and hatching capacity of 7 DSS in carp ASP with ASs

The fertilization and total hatching rates of sperm after 7 DSS in carp ASP were significantly higher than sperm stored without carp ASP (control) activated in any ASs, in which the highest fertilization and total hatching rates was over 85% and 81% with both hatchery water and Perchec solution compared to 0.2–1.6% and 0.2–1.4% in control groups (Fig. 4). On the other hand, after 7 DSS even in carp ASP, the fertilization and hatching rates decreased when 200,000 spermatozoa per egg were used. However, the hatching capacity could still reach 80% and without increased malformation rate compared to fresh sperm with a fertilization rate above 90%. In the case of using hatchery water as AS for fertilization, the worst level of malformation was obtained (3.5%), which, however, did not differ from the malformation when using fresh sperm. In general, the use of carp ASP and ASs did not affect the level of malformations (Fig. 4).

4. Discussion

The results obtained in this study indicate the efficiency and influence of the ASs on fertilization capacity and characteristics of aging sperm after storage in carp ASP for 7 days in common carp. Firstly, it showed that whenever at 15 s or 45 s PSA, hatchery water significantly increased the percentage of sperm motility than ASs with high osmolality and ions (K^+ and Na^+). Although motile sperm has been the most used motion parameter in sperm motility analyses, sperm velocities have been also considered as vital biomarkers for sperm quality and fertilization (Viveiros et al., 2010; Gallego et al., 2017). Assessment by CASA is of significant value in predicting the ability of sperm in achieving

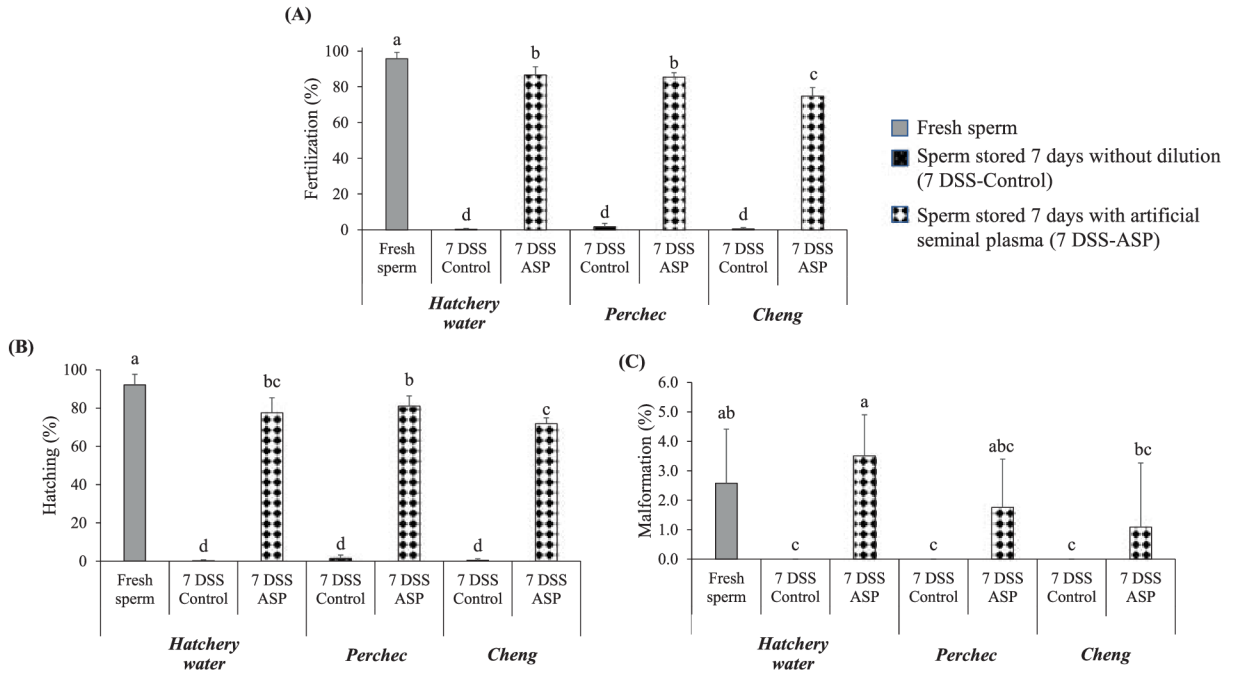

Fig. 4. . (A) Fertilization (eye stage) and (B) total hatching and (C) malformation rates (mean ± S.D.) of common carp (*Cyprinus carpio*) after storage of sperm at 7 days sperm storage (DSS) without carp artificial seminal plasma (ASP; control) and with carp ASP (Cejko et al., 2019) from six males. Sperm was activated in hatchery water, Perchec solution and Cheng solution. Fresh sperm was collected at 0 h and used as three parts of pool of sperm from each two males. The sperm for fertilization was in all cases adjusted (see Fig. 1) and used at the level 200,000 spermatozoa per egg. Values with a different lowercase letter are significantly different ($P < 0.05$).

fertilization since the percentage of motile sperm and their velocities are predictors of fertility (Rurangwa et al., 2001). It has been reported that in rainbow trout (*Onchorhynchus mykiss*) spermatozoa, had a positive correlation between fertilization rate and VCL (Tuset et al., 2008). However, in our study, the VCL and VSL in hatchery water and Cheng solution were higher than these parameters in Perchec solution. On the other hand, the only solution that was able to activate 7 DSS without carp ASP, even only at the 2% level, was the Perchec solution. During the additional studies of common carp sperm motility, we noticed that in the period of sperm storage from 0 to 3–5 days, we usually achieved a higher percentage of sperm motility using the Perchec solution. In contrast, in the later period from 3 to 5–8 days, we observed a higher percentage of motile sperm when using hatchery water. In this regard it can be assumed that a stronger osmotic shock is needed to start their activity. This also explains why fertilization and hatchability did not change when hatchery water was used to fertilize eggs compared to Perchec solution.

Siddique et al. (2016) reported that the activating medium had no impact on sperm motility parameters in the rheophileous cyprinid ide (*Leuciscus idus*), which does not correspond with our findings. It suggests that the ability of sperm to be activated is species dependent. On the contrary, Zarski et al. (2015), stated that the fresh sperm motility of common carp in AS-0 (osmolality 0 mOsm/kg) and AS-100 (osmolality 100 mOsm/kg) were significantly higher than AS-200 (osmolality 200 mOsm/kg); yet, in VCL, it was highest in AS-0 than AS-100 and AS-200. Of course, in that study, distilled water in AS-0 was tested and eggs were inseminated at different times after egg activation. In the group AS-0, the eggs had their best ability for fertilization within 60 s, but eggs kept their fertilization capacity longer (150 s in group AS-100 and 360 s in group AS-200) in higher osmolality ASs. On the other hand, although Zarski et al. (2015) only tested distilled water as AS, but in the end its osmolality and ionic level were affected by the presence of ovarian plasma from eggs at the time of fertilization. Therefore, these data must be considered as indicative. In the aged carp sperm of our study, similar results were found that hatchery water had the ability to active more sperm after short-term storage. However, higher VCL was observed in AS-4 and AS-100 than AS-130. For fertilization trial, 60 s was used, in this limited time, higher sperm motility and VCL were most important just after sperm and eggs activation. In another study of Cejko et al. (2013), it was indicated that 10 mM Tris buffer supplemented with 100 mM NaCl (pH 9.0, osmolality 199 mOsm/kg) was the most suitable for the activation of common carp fresh sperm based on sperm motility and amplitude of lateral head displacement (ALH). However, in the present study, higher osmolality can't active more sperm within 45 s after sperm storage. Consequently, it was implied that AS with higher osmolality can easily active more fresh spermatozoa rather than aged sperm after short-term storage.

Although several studies have observed the effects of AS on fresh sperm, and carp ASP effects on the sperm after short-term storage (Cejko et al., 2018, 2022), but very limited information has been obtained about the AS function on the sperm that was aged and diluted with carp ASP during *in vitro* storage. The aged sperm usually has different characteristics compared to fresh sperm. Several studies have found a considerable decline in sperm motility and velocity with storage time in several fish species (Shaliutina et al., 2013; Dietrich et al., 2021) due to a significant decrease in adenosine triphosphate content over the storage time (Aramli, 2014). It might also be associated with damage to the viability and integrity of the plasma membrane, mitochondria and sperm DNA during short-term storage (Contreras et al., 2020). On the other hand, in our study, different sperm performances in ASs with corresponding osmolality and types of extracellular ions did not cause the variation of fertilization (both 85%) between hatchery water and Perchec solution. It is important to note that, apparently the level of 200, 000 sperm per egg was probably still high to clearly show the advantage of ASs of a higher osmolality than hatchery water. Larger differences were found in another experiment when using 60,000 sperm per egg, where we were able to identify another suitable ASs for common carp in addition to Perchec solution (see supplement). On the other hand, by maintaining the sperm for 7 DSS, the percentage of motile spermatozoa decreased rapidly. Thus, the calculation of how many motile sperm were used to fertilize one egg, confirmed that the sperm count decreased rapidly. If motility of spermatozoa was calculated (1) at 15 s PSA, it was estimated to be around 19,340 spermatozoa (sperm motility 9.67% × sperm and egg ratio 200,000) and (2) at 45 s PSA, around 39,340 (sperm motility 19.67% × sperm and egg ratio 200,000) with high VCL and VSL per egg was sufficient to realize the high fertilization (85.3%) and hatching (77.5%). However, we must make it clear that the sperm count was still high, because as shown by Linhart et al. (2015) essentially 10, 000–50,000 fresh sperm per egg in common carp are sufficient for very good fertilization. If we focus on AS containing 40 mM NaCl and 20 mM Tris, after 45 s only a small amount of motile sperm was measured, which resulted in lower fertilization of 75%.

Thus, it is clear from the results obtained in our study that the use of a specific activating solution did not improve motility and fertilization in stored sperm compared to hatchery water. This phenomenon in common carp as well as European catfish, *Silurus glanis* has already been shown by Linhart et al., (2003, 2004), where the recommendation was not to use AS with high osmolality, but to use water from the hatchery and to maintain the proportions between sperm and eggs during fertilization (Linhart et al., 2020). To be noticed, the hatchery water composition may have little difference between the specific hatcheries, it should be considered in the future study. Of course, we must always take into account the fact that, in contrast to experiments with sperm motility in microscopic evaluation of the effect of AS, AS during fertilization also includes ovarian plasma, which partially increases the osmolality and ionic composition of AS.

However, the AS can influence not only sperm performance but also egg behavior. Finally, it is essential to obtain an accurate assessment of AS functions by measuring fertilization and hatching ability. This phenomenon has several possible explanations. For example, as already known, common carp eggs are strongly adhesive in fresh water. The adhesiveness of eggs, however, usually decreases when the eggs are activated by a medium characterized by a higher osmolality (Zarski et al., 2015). Sperm adhesion could be better in hatchery water than in Perchec solution, which could have a positive effect on passage of sperm through a micropyle in the egg. In addition, during artificial reproduction, the adhesive nature of the egg, apart from attaching the egg to the substratum, agglutinates the sperm to the surface of egg chorion and hinders sperm movement toward the micropyle (Siddique et al., 2016). Therefore, it could be beneficial for fertilization in the Perchec solution and achieve the similar results to the hatchery water. Aging sperm motility was found to be lower in ASs containing high osmolality than in hatchery water, but the velocity (VCL: the actual velocity along the real sperm trajectory; VSL: the straight-line distance between the start and end of the track divided by the time taken from start to finish) was similar.

Generally, the use of activating solutions with a higher osmolality than hatchery water prolongs sperm movement, but also prolongs micropyle closure (Ginsburg, 1972). The loss of fertility of eggs of common carp varies; the published data showed that the eggs in ASs with higher osmolality retain more time for opening the micropyle, which was up to 150 s in the AS-100 group and 360 s in the AS-200 group (Żarski et al., 2015). In our present study, ASs of Perchec and Cheng solution gave sperm more chance and time to fertilize eggs than hatchery water. This only manifested itself in the case of sperm without the carp ASP, although at a very small level of fertilization.

Our study also showed a great advance by storing milt for > 7 days accompanied by a higher fertilization ability of aging sperm by using ASP. There was almost 0% fertilization and hatching in groups without ASP (control). Application of an extender to store milt reduces the consumption of ATP for maintenance of vital functions and helps for ATP recovery (Ulloa-Rodriguez et al., 2018). Additionally, the

fertilization capacity of low-quality sperm was significantly elevated after incubation in ASP for 1 h (Cejko et al., 2019). No evident malformations were found in any ASs with aging sperm compared to the fresh sperm groups (fresh sperm). Therefore, our study goal to produce higher fertilization and lower malformation rates under hatchery conditions on a commercial scale was met. It is worth noting that the ratio between eggs and spermatozoa was calculated just before fertilization. The measured concentration of aging sperm should have been accurate because it was measured just prior to fertilization (Cheng et al., unpublished data and Dietrich et al., 2021). As documented by Cheng et al. (unpublished data) after 8 DSS, the sperm of common carp is reduced to 50% if it is not diluted with carp ASP and by 22% if it is diluted. Therefore, before fertilization itself, it is necessary to consider this phenomenon in common carp and increase the number of aged spermatozoa used for fertilization compared to the same samples without storage. As was demonstrated in the present experiment, if the quantity of sperm for fertilization is adjusted, then 85% fertilization is ensured even under conditions in a hatchery for common carp.

5. Conclusions

This study suggested the feasibility of using aging sperm previously collected and diluted with ASP in artificial reproduction on a commercial scale. Aging sperm concentration should be measured just before application to guarantee there is sufficient sperm to provide fertilized embryos and for larvae production. Thus, it is possible to use aging sperm in common carp within 7 days efficiently at 4 °C and achieve high fertilization rate compared to sperm without carp ASP. Finally, according to the results from our present study, the carp ASP, hatchery water and Perchec solution could be used in common carp aquaculture.

CRediT authorship contribution statement

Y.C. and O.L. conceived and designed the experiments; Y.C., SP.Z., and Z.L. carried out the evaluation of semen quality and performed statistical analysis of the data; Y.C. and O.L., wrote the manuscript; Y.C., SP.Z., Z.L., N.E.S. and O.L. contributed to revising and reviewing the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2022.101160

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CHAPTER 6

CHANGES IN PHENOTYPES AND DNA METHYLATION OF *in vitro* AGING SPERM IN COMMON CARP, *Cyprinus carpio* L.

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My share on this work was about 60%.

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Article

Changes in Phenotypes and DNA Methylation of In Vitro Aging Sperm in Common Carp *Cyprinus carpio*

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Abstract: The purpose of the current study was to analyze phenotypic and functional characteristics of common carp (*Cyprinus carpio*) spermatozoa during in vitro aging and to investigate whether global DNA methylation is affected by sperm aging. Milt was collected from five individual males, stored in vitro on ice in a refrigerator for up to 96 h post stripping (HPS) and used to fertilize eggs with intervals of 1, 24 and 96 h. Computer-assisted sperm analysis and a S3e Cell Sorter was employed to determine the spermatozoa phenotypic characteristics (motility, velocity, concentration and viability). In addition, pH and osmolality of the seminal fluid and the capacity of the spermatozoa to fertilize, hatching rate and health of the resulting embryos were examined at different aging times. Whole-genome bisulfite sequencing was used to compare the global and gene-specific DNA methylation in fresh and aged spermatozoa. The results demonstrated that spermatozoa aging in common carp significantly affects their performance and thus the success of artificial fertilization. The methylation level at the cytosine-phosphate-guanine (CpG) sites increased significantly with 24 HPS spermatozoa compared to the fresh group at 1 HPS and then decreased significantly at 96 HPS. A more detailed investigation of gene specific differences in the DNA methylation was hindered by incomplete annotation of the *C. carpio* genome in the public databases.

Keywords: fish; common carp; sperm aging; epigenetics; DNA methylation; sperm quality; milt; fertilization; sperm storage

1. Introduction

Fish spermatozoa quality depends on the breeding season, age of the males and manipulation of the gametes in vitro [1,2]. Declining spermatozoa quality is directly associated with a decrease in spermatozoa performance resulting in poor fertilizing ability and the quality of the resulting offspring [3,4]. Moreover, a decline in sperm quality leads to a decrease in energy stores and changes in proteins [5]. Alterations in DNA and membrane integrity, and mitochondrial membrane potential have also been noted [2,6–9]. Sperm aging is accompanied by decreased spermiation, sperm motility and velocity, as well as modification in seminal plasma composition, and oxidant and antioxidant level changes [6,10]. The mechanism of sperm aging is thus important in reproduction. The free

radical theory of aging [11] suggests that oxidative stress due to excessive free radicals or reactive oxygen species is responsible for the aging process [12]. However, recent studies indicate that oxidative stress is probably not the main promotor of the aging process in fish oocytes [13]. Epigenetics including DNA methylation are suggested as one of the important mechanisms behind the aging process [14].

DNA methylation and demethylation patterns are established by DNA methyltransferase (DNMTs) enzymes, that contain DNMT1 and DNMT3 with two distinct functions [15]. DNA methylation in spermatozoa has been related to sperm quality. For example, the level of sperm DNA methylation has a significant positive correlation with male reproductive capacity in striped bass (*Morone saxatilis*) [16] and sperm concentration and motility in humans [17]. There are many studies on DNA methylation profiles in mammalian sperm [18–22]. Abnormalities of DNA methylation caused by modifications in cytosine phosphate guanine (CpG) islands and shore regions result in increased susceptibility to disease, abnormalities and disorders in the offspring from more aged mice sperm [23]. Mice sperm aging causes high risks of aberrant glucose metabolism and the development of autism spectrum disorders and behaviors in intergenerational and transgenerational offspring [18]. Sperm DNA methylation in mice has also shown to be responsive to abnormal/functioned physiology [19,20]. However, some other studies reported that no imprinted genes of DNA methylation were detected in sperm of obese human [21] and cryopreserved sperm in human [22].

A fish is a suitable model animal for conducting a study on sperm aging because it displays a vast diversity of reproduction modes, and the milt contains about 1000 times more spermatozoa per ml than mammals [24]. Obtaining a large number of spermatozoa can be synchronized by modulation of the external environment such as hormonal treatment. The additional advantage is that fish spermatozoa are immotile at the time of collection, allowing easier investigation of aging over a long-time period of up to several days of storage. The motility of spermatozoa is activated only after exposure to water or suitable activating media [25]. We chose common carp for this study because it produces a large number of gametes within a single reproduction season, i.e., around 2 kg of eggs (1.6 million eggs) and 30 mL of sperm (600×10^9 spermatozoa) can be produced by an individual female or male, respectively [26,27].

The aim of the present study was to monitor the changes in sperm phenotypic parameters (motility percentage, velocity, concentration, viability; pH and osmolality of seminal fluid; fertilization, hatching and malformation rates) and its relation to DNA methylation levels in common carp during in vitro spermatozoa aging. We hypothesized that sperm aging induces changes in sperm DNA methylation levels and other relevant sperm kinetic functions and fertilization parameters. Our present study is the first report that allows a better understanding of possible epigenetic alterations during the aging of common carp spermatozoa.

2. Results

2.1. Effects of Pooled Sperm In Vitro Aging on Different Phenotypic Characteristics

Spermatozoa motility measured at 15 s post sperm activation (PSA) decreased rapidly from 88.1% at 1 h post stripping (HPS) to 61.0% at 24 HPS, then to 59.5% at 48 HPS and finally 36.9% at 96 HPS (Figure 1A). Once the percentage motility of spermatozoa was analyzed in detail, focusing on fast, medium, slow and static spermatozoa (vibration in-situ) (Figure 1B), 81.7% of fast spermatozoa were recorded at 1 HPS. Subsequently, there was a sharp decrease to 49.8% of fast spermatozoa after 24 HPS and 23.9% after 96 HPS.

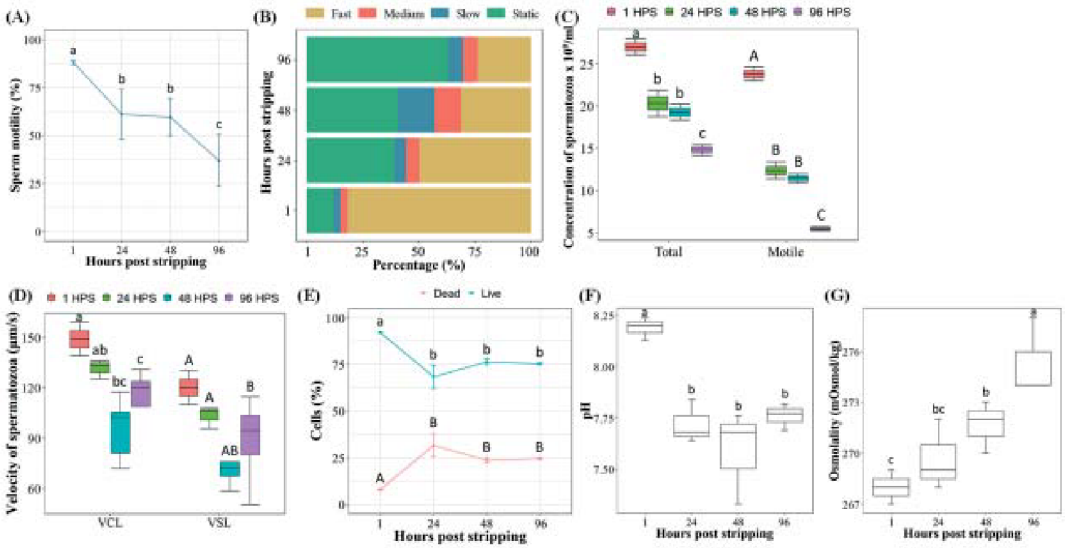

Figure 1. Effect of pooled milt aging (5 males) stored at 1, 24, 48 and 96 h post stripping (HPS) in common carp (mean ± S.D.): (**A**) percentage of sperm motility evaluated at 15 s of post sperm activation (PSA); (**B**) percentage of sperm motility from total motility of spermatozoa evaluated at 15 s of PSA: rapid motility (>100 μm/s), medium motility (46 to 100 μm/s), slow motility (10 to 45 μm/s) and static spermatozoa (<10 μm/s); (**C**) total and motile sperm concentration per mL; (**D**) curvilinear velocity (VCL) and straight line velocity (VSL) evaluated at 15 s PSA; (**E**) viability of spermatozoa cells; (**F**) pH of seminal fluid; (**G**) osmolality of seminal fluid. Values with different letters within lower- or upper-case letters, are significantly different [$p < 0.05$, one-way analysis of variance (ANOVA) followed by an LSD test for post hoc multiple comparisons].

During milt storage, a relatively significant spermatozoa loss was found (Figure 1C). At 1 HPS, spermatozoa concentration was 27.0 × 10^9/mL spermatozoa. Subsequently, there was a reduction to 20.3 × 10^9/mL spermatozoa after 24 HPS and finally after 96 HPS only 14.8 × 10^9/mL of spermatozoa remained in the milt. Figure 1C is supplemented by values of the number of motile spermatozoa from the concentration of spermatozoa per ml, which showed a rapid decrease in the number of motile spermatozoa per ml. Initially, 23.8 × 10^9 motile spermatozoa per ml were recorded. Thereafter, there was a sharp decrease to about half after 24 HPS, and then only 11.5% of the motile number of spermatozoa per ml remained (5.5 × 10^9 motile spermatozoa per ml) after 96 HPS.

The curvilinear velocity of spermatozoa also gradually slowed with increasing storage time (Figure 1D). The reduction in sperm curvilinear velocity (VCL) was not as abrupt as that of the sperm motility rate. At 15 s PSA, spermatozoa VCL was 148.8 μm/s at 1 HPS, then VCL decreased to 131.4 μm/s at 24 HPS. There was a significant decrease in VCL to 95.6 μm/s after 48 HPS and curiously an increase in VCL of spermatozoa to 112.0 μm/s after 96 HPS was noted. Relatively high levels of slow and moderately slow spermatozoa (medium speed) occurred at 48 HPS compared to other HPS (Figure 1B). In the case of straight-line velocity (VSL), a similar trend of a decrease up to 48 HPS with a following increase at 96 HPS was noted.

The highest value of sperm viability with 92.0% live spermatozoa was observed in fresh milt (1 HPS), and a significant decrease in live spermatozoa occurred at 24 HPS (68.3%). Subsequently, the sperm viability stayed stable around 75.83 ± 1.07% live spermatozoa at 48 and 96 HPS (Figure 1E).

The pH and osmolality of the seminal plasma also varied significantly through the milt storage time (Figure 1F,G). The change in pH was significant, decreasing from the initial value of 8.2 at 1 HPS to 7.6 and 7.8 at 24 and 96 HPSs, respectively. In contrast, the osmolality increased significantly from 268.0 mOsmol/kg at 1 HPS to 275.3 mOsmol/kg at 96 HPS.

2.2. *Effects of Sperm In Vitro Aging from Individual Males on Phenotypic Characteristics*

Sperm from the five individual males was used to confirm the validity of the phenotypic parameters achieved in the previous experiment in the pooled aging milt (Figure 2A–F). Motility of spermatozoa rapidly decreased from the initial value of 89.3% at 1 HPS to 28.2% at 96 HPS (Figure 2A). The proportion of fast-moving spermatozoa was measured as *c* 80.5% in the freshly collected milt, while at 96 HPS, only 17.7% was fast-motile spermatozoa (Figure 2B). In addition, sperm concentration showed a sharp decrease from the initial value of 23.8 × 10^9/mL spermatozoa to only 16.3 × 10^9/mL after 96 HPS, which represented a decrease in sperm concentration per ml of 31.2% (Figure 2C). The number of motile spermatozoa per ml decreased even more quickly, from 21.1 × 10^9/mL to <4.8 × 10^9/mL at 96 HPS (Figure 2C). The VCL and VSL of spermatozoa similarly decreased from values of 134.6 μm/s and 110.4 μm/s at 1 HPS to 94.2 μm/s and 74.5 μm/s at 96 HPS, respectively (Figure 2D). The proportion of spermatozoa activity with the medium and slow speed groups (Figure 2B) was similar at different HPSs, therefore the sperm velocity showed a balanced decrease (Figure 2D).

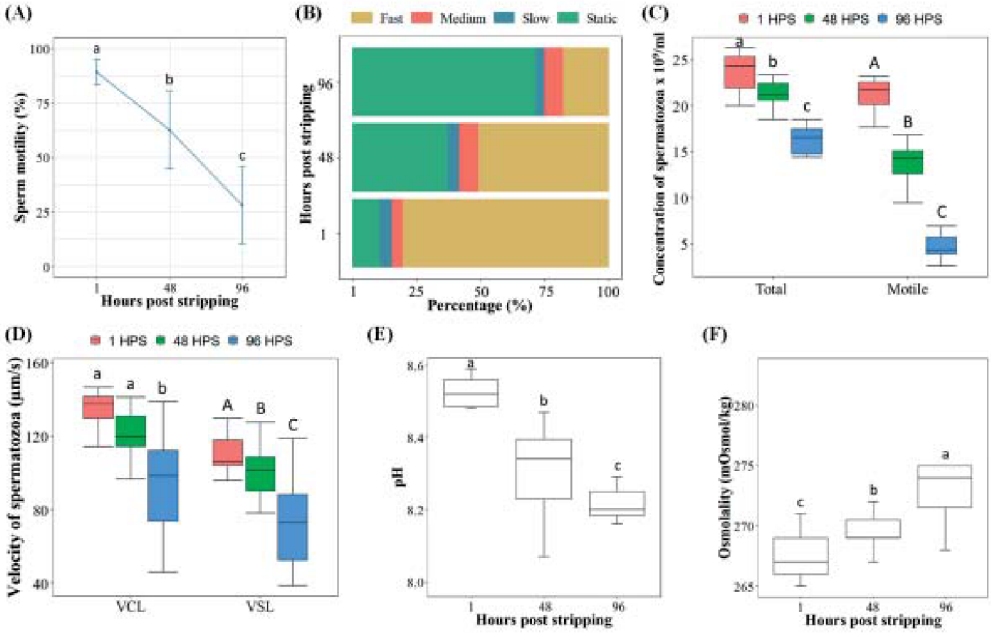

Figure 2. Effect of aging milt from five individual males stored at 1, 48 and 96 HPS in common carp (mean ± S.D.): (**A**) percentage of sperm motility evaluated at 15 s of post sperm activation (PSA); (**B**) percentage of sperm motility from total motility of spermatozoa evaluated at 15 s of PSA [rapid motility (>100 μm/s), medium motility (46 to 100 μm/s), slow motility (10 to 45 μm/s) and static spermatozoa (<10 μm/s)]; (**C**) total and motile sperm concentration per ml; (**D**) curvilinear velocity (VCL), straight line velocity (VSL) evaluated at 15 s of PSA; (**E**) pH of seminal fluid; (**F**) osmolality of seminal fluid. Values with different letters within lower or uppercase letters are significantly different ($p < 0.05$, one-way analysis of variance (ANOVA) followed by an LSD test for post hoc multiple comparisons).

At the beginning of the experiment, seminal plasma had a pH of 8.52 and at the end, after 96 HPS, it still kept a relatively high pH value of 8.21 (Figure 2E). Additionally, osmolality increased significantly with time, increasing from 267.2 mOsmol/kg at 1 HPS to 274.5 mOsmol/kg after 96 HPS (Figure 2F).

2.3. Effects of Pooled Sperm In Vitro Aging on Fertilization, Hatching and Larval Malformation Rates

The results from the freshly collected sperm at 1 HPS showed that 31,250,000 spermatozoa were sufficient to obtain 79.9–97.5% fertilization and 57.4–91.9% hatching rates (Figure 3A,B). The fertilization and hatching rates of the aging sperm at 24 HPS decreased by 30.6% compared to the control at same HPS. The number of 31,250,000 sperm stored for 24 h was able to ensure fertilization and hatching at the level of 66.9% and 51.8%, respectively. Sperm fertility decreased rapidly at 96 HPS. Compared to the controls at the same HPS, fertility and hatching rates were reduced 75.7% and 71.9% at 96 HPS, respectively. Malformation rates of embryos from 24 and 96 HPS aged sperm groups (0–9.12%) were lower or not significant compared with control groups at the same HPS (Figure 3C).

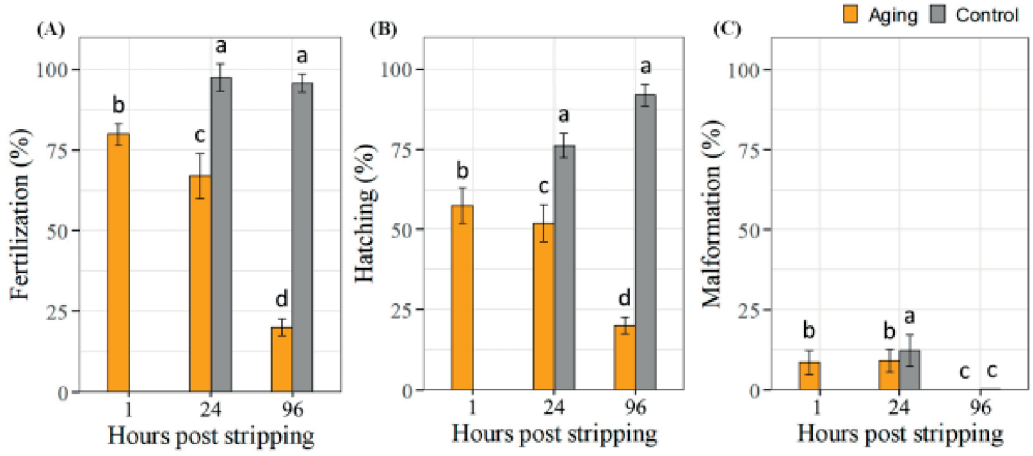

Figure 3. (**A**) Fertilization (eye stage), (**B**) hatching and (**C**) malformation rates (mean ± S.D.) of common carp with a total number of 31,250,000 spermatozoa. Aged milt was stored for 1, 24 and 96 h individually and pooled just prior to fertilization from four males. Controls were fresh milt collected at 24 and 96 h and used as a pool of milt from three males. Eggs were also pooled from four females. Values with the different superscript are significantly different ($p < 0.05$).

2.4. Bisulfite Sequencing of Carp Spermatozoa Genomic DNA during Aging

The DNA methylation patterns in freshly collected and aged carp sperm were investigated at 1, 24 and 96 HPS. We performed the whole-genome bisulfite sequencing (WGBS) of genomic DNA of the three samples, each represented by three biological replicates, and we received on average 8.67× sequencing coverage of the carp genome assembly GCA_000951615.2 (1.7 Gb). The WGBS sequencing statistics and quality filtering statistics are listed in the Supplementary Table S1.

Trimmed reads were mapped to the reference using the Bismark Bisulfite Read Mapper with an average 71% mapping efficiency. Bismark PCR deduplication script detected and removed on average 18% of the mapped reads. The details of the mapping and deduplication statistics are given in Supplementary Table S2. Context-dependent methylation calls were extracted using a Bismark methylation extractor. Cytosine methylation calls were extracted for different nucleotide contexts and methylomes of all three libraries were compared. The details of cytosine methylation calls are given in Supplementary Table S2. The ratio and extent of cytosine methylation in different nucleotide contexts show values typical for a vertebrate genome with the prevalence of CpGs methylation (Figure 4) and thus the differential methylation was done with methyl-CpG (mCpG) context only. Interestingly, the cytosine methylation report revealed that the level of mCpG fluctuates at 24 HPS aged spermatozoa (Figure 4) in comparison to 1 and 96 HPS.

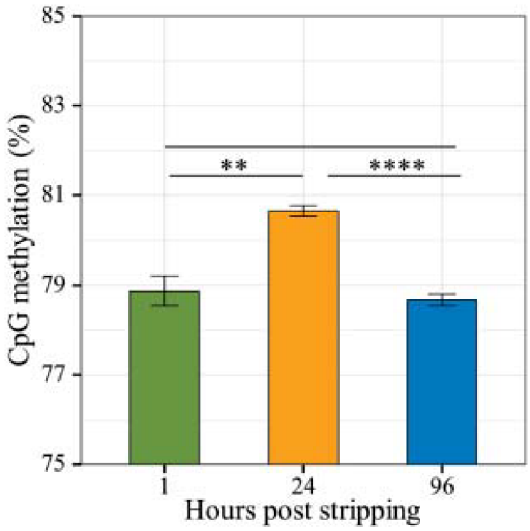

Figure 4. Percent change of differentially methylated regions (DMRs) among the three libraries. The DMRs were calculated by Defiant, and each value is statistically supported by three biological replicates. Asterisks denotes *p*-value between two groups (** $p < 0.01$, **** $p < 0.0001$).

2.4.1. Differential Methylation Analysis

Methylation calls of the three libraries were submitted for a differential methylation analysis by Defiant using pairwise comparison of 1 HPS and 24 HPS libraries, and 1 HPS and 96 HPS libraries. The details of the Defiant run are listed in Supplementary Table S3. A total of 1305 and 1729 differentially methylated regions (DMRs) were detected by Defiant in 24 and 96 HPS libraries, respectively. These two comparisons aimed at describing the gradual changes in cytosine methylation over the 4 days of carp sperm storage, using the 1 HPS library as a reference for the calculation of DMRs in both 24 and 96 HPS libraries. The number of mCpGs increased by 1.76% ($p = 0.00376$) at 24 HPS DNA from 78.9% at 1 HPS to 80.7% at 24 HPS and decreased to 78.7% at 96 HPS, which represents only an insignificant decrease of only 0.27% ($p = 0.32257$) in comparison to the 1 HPS library. This interesting DMR fluctuation over the time of sperm aging encouraged us to calculate the pairwise comparison of mCPG counts for 24 and 96 HPS libraries using the 24 HPS library as a reference and the 96 HPS library for DMR inference. In this comparison we observed a significant decrease of mCpGs by 1.97% ($p = 0.00001$) (Figure 4).

Closer investigation of the DMRs fluctuations revealed that 38 DMRs that underwent methylation at 24 HPS were found unmethylated at 96 HPS while only 3 DMRs experienced the opposite process, i.e., they were unmethylated at 24 HPS and methylated at 96 HPS. The identity of these DMRs is given in Supplementary Table S4.

2.4.2. Functional Annotation of Differentially Methylated Loci

Prior to functional annotation, a re-annotation of DMRs in both 24 and 96 HPS libraries was done in order to populate an incomplete annotation record of the carp genome and identify missing transcripts and protein IDs for chromosomal loci that are actively transcribed. Additionally, missing protein IDs for transcript IDs already present in the carp genome were also collected. A summary of DMR statistics is given in Table 1.

Table 1. An annotation summary of differentially methylated regions (DMRs) in both 24 and 96 h post stripping (HPS) libraries. Only unequivocally assigned gene accessions and DMRs having a >95% sequence identity hit in the common carp transcriptomic data were considered in the list of genes. The remaining DMRs with no available evidence of expression were included in the list of putative intergenic regions.

Library	CpG Methylation State	DMRs	No. of Genes	No. of Intergenic Regions
24 HPS	methylated	920	123	797
	unmethylated	385	258	127
96 HPS	methylated	875	300	575
	unmethylated	854	288	566

A complete list of DMRs, including statistically supported counts of DMRs calculated by Defiant and their annotation records are given in Supplementary Tables S4–S7 for pairwise comparisons of 1 × 24, 1 × 96 and 24 × 96 HPS libraries, respectively.

Functional annotation and GO enrichment of DMRs at 24 and 96 HPS were performed in order to unveil a potential linkage of CpG methylation and sperm aging. A direct link of CpG methylation with groups of genes employed in specific cellular processes suggests a regulatory function of CpG methylation in the deterioration of spermatocyte viability and/or motility. Unique protein IDs for DMRs from 24 and 96 HPS libraries were submitted to GO annotation with InterProScan and the assigned GO terms were further used for functional enrichment by GOATOOLS. Annotations could be assigned to 285 and 449 unique protein IDs from the 24 and 96 DMR subgroups, of which 186 and 281 were assigned a GO term, respectively. Only a single GO term was found significantly enriched after a false discovery rate correction in the 24 HPS dataset.

3. Discussion

The results obtained demonstrated that the quality of spermatozoa in common carp is significantly affected by the time of in vitro storage. The motility rate and the live spermatozoa notably decreased within the first 24 HPS together with pH of the seminal fluid. Fertilization rate also decreased from 90% to 67% at 24 HPS. These results are consistent with previous studies on other fish species, although there is considerable variation in storage capacity of sperm between species. For example, the rainbow trout (*Oncorhynchus mykiss*) sperm could be stored for 34 days [28], while zebrafish (*Danio rerio*) sperm stored in Hank's balanced salt solution were viable for <1 day [29].

Sperm motility is considered to be one of the most useful parameters to determine sperm quality, which is highly correlated with fertilization success [30]. In the present study, a sharp decrease in sperm motility was observed at 24 HPS for pooled sperm. Such a sharp decreasing trend in sperm motility was also recorded in the experiment using individual male spermatozoa. The lowest motility rates were recorded as 36.75% and 28.23% at 96 HPS for both the pooled and individual sperm storage experiments, respectively. In addition to the reduction in sperm motility and velocity, a decrease in total spermatozoa concentration was observed with increasing storage time. After 96 HPS, only 55% of the original total spermatozoa number in ml remained in the pooled sperm and only 69% in the sperm from individual males. However, these data do not usually appear in the fish spermatology literature. Only Dietrich et al. [31] reported a similar result in common carp sperm with a relatively large reduction of spermatozoa number per ml after 72 HPS. The common carp spermatozoa die and disintegrate with increasing milt storage time. In our experiment, the highest changes in seminal plasma after 24 h were recorded with a significant decrease of pH and also with increasing osmolality.

Not only the number of spermatozoa but also the number of motile spermatozoa were significantly affected by the storage time. At 96 HPS, only 20% of spermatozoa remained motile. It was also found that fast spermatozoa predominate at 1 HPS and the most spermatozoa with static movement were detected at 96 HPS. Based on these results, at 1, 24, 48 and 96 HPS about 16–19 × 10^9, 6 × 10^9, 4–7 × 10^9 and 1–1.4 × 10^9 spermatozoa within

the fast movement category were recognized, respectively, for both mixed and individual sperm experiments (if a reduction in sperm count is taken into account). The velocity and motility of spermatozoa movement determines the success of sperm competition and fertilization [24,25,32]. In a previous study [33], high correlations were found between these parameters and fertilization and hatching rates, Similar results have also been reported in Atlantic halibut (*Hippoglossus hippoglossus* [34]) and common carp [35]. Usually in freshwater bony fishes the spermatozoa motility and the time of closure of the micropyle in the egg is very short. Therefore, very fast-moving spermatozoa are pre-requisite, so that they can fertilize the egg within 20–30 s [32]. In common carp, the spermatozoa can move for a maximum of 2 min. Nevertheless, rapid movement has been recorded only up to 30 s [26,36]. A previous study conducted on Indian major carp orangefin labeo (*Labeo calbasu*) have also observed a similar significant decrease in sperm motility during the first 12 h of storage [37]. The energy source [adenosine triphosphate (ATP) is the most common] produced and stored during the quiescent stage of spermatozoa in seminal plasma has been implicated as the primary source of immediate energy needed for spermatozoa motility [38,39]. Reduction in sperm motility is assumed to be a result of a decrease in ATP and oxygen during the storage time [40]. Motility decrement depends on the impairments in various other aspects of the cell, such as the physiological state of the mitochondria, plasma membrane and DNA integrity and flagellum structure [24,32].

The fertilization rate was recorded above 90% in the fresh control groups at 24 and 96 HPS and around 20% in the aging group at 96 HPS when over 450,000 spermatozoa per ova were used. The rapid decrease observed in fertility after 96 HPS could be due to a sharp decrease in the number of fast-moving spermatozoa. Out of the total of 450,000 spermatozoa, 250–350,000 spermatozoa with fast movement were found in fresh sperm. However, at 96 HPS, the number of fast-moving spermatozoa was reduced to 20,000 from the rest of the total (see reduction in Figure 1A–C).

The motility of cyprinid sperm indicates their fertilization capacity [41]. In our present study, the velocities (VCL, VSL) of stored sperm also gradually slowed down with increasing storage time but not as fast as the sperm motility rate. Therefore, we verified the theory given by Taborsky [42] that sperm traits such as swimming speed and motile sperm are one of the factors which results in reduction of fertilization during sperm aging [42]. In practice, it is possible to compensate for the reduction of fertility due to a decrease in the percentage with good sperm velocity and motility by increasing sperm quantity. If good fresh sperm (motility above 80%) is stored over 96 h, around 20 times more spermatozoa would need to be used from the initial level. Therefore, to improve the fertilization rates after 96 HPS, a sperm use level of at least 10^6 spermatozoa will be required.

Common carp under natural conditions fully spermiate during June to September, when the water temperature of the ponds reaches 20–21 °C. Under natural conditions, the volumes of milt produced by the males are increased by the presence of ovulating females in the ponds [43,44]. The best quality was observed in samples obtained at the middle of the natural spawning season [44–46]. However, some spermiation can be obtained with fish kept isolated in experimental tanks if the water temperature is above 18–20 °C. In that situation, hormonal stimulation significantly increases the volume of milt available (five to ten-fold) and ensures sperm production [47,48]. Studies on the motility parameters reflecting carp sperm qualities (concentrations of spermatozoa and percentages of motile cells) indicated that the quality of sperm obtained without hormonal stimulation is superior to that of samples obtained after hormonal injection. The sperm we subjected to total DNA methylation showed us that the level of mCpG fluctuates in 24 HPS aged spermatozoa (Figure 4) in comparison to 1 and 96 HPS. Rather, the largest changes were expected after 96 HPS. In addition, the increase in DNA methylation negatively correlates with parameters used for the evaluation of spermatozoa quality. The observation is consistent with numerous studies that put into context aberrant sperm quality parameters and changes in mCpG levels in aged and/or infertile males [17,49,50]. Surprisingly, the majority of these studies, which also include other vertebrate species, observed an increase [51–55]

rather than a decrease [23] in global DNA methylation. The DNA methylation change after 24 HPS can be explained in relation to the environmental changes that have occurred by the spermatozoa. As mentioned above, sperm volume increased enormously after hormonal injection with varying levels of spermatozoa quality being released from the cysts in the testes. In addition to young spermatozoa, there were also older and old populations of spermatozoa. The existence of different populations with possible separation in sperm has been demonstrated in carp [56]. Stripping of sperm into the external environment and storage in vitro at 0–2 °C resulted in a new physiological state that caused a change in DNA methylation in a portion of the population at approximately 24 HPS. However, sperm with changes in DNA methylation soon died and were no longer detectable after 96 HPS. This was evident by a sharp reduction in the sperm count after 96 HPS, including pH stabilization.

Research related to fish breeding management has also greatly advanced and the search for a good molecular diagnostic marker describing fish sperm quality is now being recognized and more appreciated [57–59]. The data collected in our study were used to compare epigenetic changes in carp sperm DNA with established sperm phenotypic parameters in fresh and aged carp sperm. This comparison gave us an opportunity to identify genomic loci whose hypermethylation is typical for aberrant sperm populations and hence have a potential to be used as epigenetic markers. This also facilitates the diagnostics of low-quality carp sperm and introduces improved techniques for sperm storage and handling in carp breeding programs. Similar research was conducted in a previous study focused on the correlation of DMRs in high-fertility and low-fertility striped bass individuals [16]. The comparison of the two study groups revealed 171 DMRs and functional annotation of these DMRs uncovered two important groups of genes that are related to the striped bass fertility performance. In our study, we performed the GO enrichment of annotated DMRs, however, no enrichment was found in either of the study groups that were compared. It is important to note that the design of our present study and the study conducted on the striped bass are not really comparable. Additionally, the global methylation analysis in the study of striped bass was done using the Methyl-CpG binding domain sequencing (MBD-seq) method while our study used the WGBS method. It is interesting to note, however, that both low fertile spermatozoa in the striped bass and low-quality aged spermatozoa in carp are both associated with changes in DNA methylation levels. The study of the DMRs in low fertile striped bass found a clear link between the DMRs identified and the genes involved in fish fertility. In our study, we did not find any functional enrichment. One of the major limitations of our findings is the lack of a well annotated genome, which could cause the omission of important genes that would otherwise contribute to the possible enrichment in certain functional group of genes. It is thus of major importance to perform a more thorough annotation of the DMRs in the future.

On the contrary, the study of the methylome in bad and good breeders in zebrafish showed no difference in methylation levels between the two study groups [59]. However, it is important to note that the analysis of differential methylome in this study was conducted using the methylation specific restriction digestion method which is by orders of magnitude less sensitive and specific then WGBS or MBD-seq and does not provide any information about methylation status of specific CpGs. The locus specific analysis of methylation was done in the promoter of the *dmrt1* gene, which is a key regulator of male sex determination in vertebrates and is particularly useful in fishes that lack sex determination by distinct sex-specific chromosomes [60]. Similarly, this locus specific methylation analysis did not show any significant difference in methylation between good and bad breeders of zebrafish. Due to the inconsistency in study designs as well as in methodological approaches, these three studies cannot be fully compared, and more extensive and thorough research need to be conducted in order to make any valid conclusions about the methylation as a reliable marker of fish sperm quality and the effect of sperm aging.

The effect of altered DNA methylation profiles in aberrant spermatozoa are often linked to the abnormal differential expression that occurs during spermatogenesis. Since

the mature spermatozoa are transcriptionally inactive due to the highly condensed state of their chromatin [61], it is possible that the hypermethylation during sperm aging is driven by the set of transcripts carried by a mature spermatozoon, whose abundance was changed during spermatogenesis. This aspect has also been addressed by studies investigating the transcript identity and abundance during spermatogenesis in mature spermatozoa [62,63].

It is hypothesized that the representation and abundance of transcripts, including transcripts for methyltransferases, could be influenced by genetic failure or external stress stimuli during spermatogenesis [64]. Poor quality parameters and lowered viability of spermatozoa are often associated with environmental stress that is believed to be manifested in the observed phenotypes via epigenetic regulation. The effect of environmental changes on the altered gene expression are largely implemented by the changes in the epigenome, which further interacts with a networks of DNA methylation reader proteins playing a role in a cascade of gene expression regulation. This assumption led to the identification of a sperm-specific catalogue of transcripts and characterization of gene expression profiles in normal [65,66] and infertile sperm in vertebrates which yielded several promising candidate transcripts that are currently in use as markers of male sperm quality and fertility [50,62,67–69]. The methylation changes that occurred in the subvital and aberrant sperm cells at 24 HPS might have been driven by the changes in the gene expression and thus by altered representation of transcripts that could have led to the observed hypermethylation with subsequent deterioration of sperm quality traits and eventual death of these cells.

Another factor that could play a role in the observed DNA hypermethylation with subsequent death of aged sperm cells could be the sperm sample handling procedure and subsequent storage conditions. Upon stripping, the milt samples were transferred directly into ice, which represents a 20 °C difference in comparison to the body of the carp males. Such a change in the environmental temperature could be considered a shock for the sperm cells and could thus contribute to the induction of methylation changes in the part of the susceptible sperm cell population. The mechanism driving these changes in response to environmental stress remains to be elucidated. The effect of sperm cryopreservation on the differential methylation changes has been investigated before in zebrafish [70]. Interestingly, specific regions of zebrafish genome were hypermethylated, but others were not affected. Additionally, the follow up study found that only subpopulations of spermatozoa experienced hypermethylation while others were hypomethylated.

Our data, however, require further investigation. A more thorough carp genome annotation should allow identification of other types of genomic features such as gene promoters and genomic repeats which are both frequent targets of cytosine methylation and often play a crucial role in gene expression, genome architecture or silencing of transposon activity.

4. Material and Methods

4.1. Ethics Statement and Animals

The study was conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Vodňany, Czech Republic. The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016–17214). The expert committee of the Institutional Animal Care and Use Committee of the FFPW approved the methodological protocol of the current study according to the law on protecting animals against cruelty (reference number: MSMT-6406/2019-2).

4.2. Experimental Fish

The research was carried out at the Research Institute of Fish Culture and Hydrobiology of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Broodstock handling was performed according to Linhart et al. [71]. Before injection and gamete collection, the males and females were anaesthetized in a solution

of 2-phenoxyethanol (1:1000). The best quality eggs (darker coloration, without excessive ovarian fluid and no apparent decomposition) were visually selected from individual females and immediately used for the fertilization assay.

4.3. Experimental Design

After collection, spermatozoa were cooled down at 0–2 °C and motility was evaluated microscopically; sperm samples with a minimal motility of >90% were chosen for all experiments. Experimental Eppendorf tubes (Sigma-Aldrich, Taufkirchen, Germany) used for storage of milt were kept as 1200 µL of air and 600 µL of milt (ratio air: milt = 2:1) and the lids kept closed.

4.3.1. Phenotypic Parameters of Pooled Sperm during In Vitro Aging

Milt from five males from a recirculation aquaculture system (RAS) was pooled, distributed in four 2 mL tubes and stored on ice in a refrigerator under aerobic conditions for 1, 24, 48 and 96 h. The motility, velocity, viability and concentration of stored spermatozoa, and pH and osmolality of seminal fluid were recorded in triplicate at each studied time point (1, 24, 48 and 96 HPS).

4.3.2. Phenotypic Parameters of Sperm from Individual Males during In Vitro Aging

Milt from five individual males from RAS was distributed in fifteen 2 mL tubes (three per male) and stored on ice in a refrigerator under aerobic conditions for 1, 48 and 96 h. The motility, velocity and concentration of stored spermatozoa as well as pH and osmolality from seminal fluid were recorded three times at 1, 48 and 96 HPS.

4.3.3. Fertilization and Hatching Level with Malformation Embryos Rates from Pooled In Vitro Aged Sperm

The milt samples from five males from a pond were pooled and stored on ice in three 2 mL tubes under aerobic conditions. Fertilization experiments with pooled milt were performed at 1, 24 and 96 HPS. Freshly ovulated eggs were pooled from three females for each milt storage time of 1, 24 and 96 HPS. In addition, pooled freshly collected control milt from three males from a pond were used to test fertilization at 24 and 96 HPS. Prior to fertilization, the concentration of pooled sperm from short-term storage at 1, 24 and 96 HPS was 19.06×10^9 spermatozoa per ml, and pooled fresh control sperm at 24 and 96 HPS was determined at 20.53 and 27.40×10^9 spermatozoa per ml, respectively.

4.3.4. Whole Genome Bisulfite Sequencing from Pooled In Vitro Aged Sperm

Milt from five males from RAS was pooled, distributed in three 2 mL tubes and stored on ice in a refrigerator under aerobic condition for sampling at 1, 24 and 96 HPS.

4.4. Examining Phenotypic Characteristics of Spermatozoa during In Vitro Aging

4.4.1. Sperm Motility, Velocity and Concentration

Distilled water supplemented with 0.25% Pluronic F-127 (catalog number P2443, Sigma-Aldrich; used to prevent sperm from sticking to the slide) was used as an activating medium (pH 7.0–7.5), that was kept on ice during the whole experiment. Spermatozoa were activated at room temperature (21 °C) by mixing the diluted sperm sample with needles into 10 µL of the activation medium on a chamber SpermTrack-10® (Proiser R + D, S.L.; Paterna, Spain) at different times of storage. The activated spermatozoa were recorded microscopically and followed the techniques described previously [72]. Computer-assisted sperm analysis included the percentage of motile sperm (%), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s) and spermatozoa rate with rapid motility (>100 µm/s), medium motility (46 to 100 µm/s), slow motility (10 to 45 µm/s) and static spermatozoa (<10 µm/s). Scales in sperm analysis software were calibrated and the base on the microscope and adaptor set. Escala X and Escala Y were both set up to 1.38 µm

when using 10 × lens on a negative phase-contrast condenser microscope. Quantitative analyses of all samples were conducted in triplicate.

Sperm concentration together with the total number of sperm per male and mixed sperm from five males was evaluated at different HPS. The sperm concentration (expressed as 10^9 spz/mL) was determined by a Bürker cell hemocytometer (Marienfeld, Germany, 12 squares counted for each male) using an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) under an Olympus microscope BX 41 (4009). For counting the cell number clearly, the Bürker cell hemocytometer containing sperm samples was placed horizontally for about 3 min for the cells to sediment.

4.4.2. Osmolality and pH of Seminal Plasma

Sperm samples were centrifuged at 17,000× *g* at 4 °C for 15 min twice to separate the cell and supernatant (Thermo Scientific, Fresco 21; Thermo Scientific, Walthan, MA, USA). From the seminal plasma collected (supernatant), osmolality from 50 µL and pH from 250 µL samples were determined. Data for osmolality were collected in triplicates by using a Freezing Point OSMOMAT 3000 (Gonotec, Germany) and expressed in mOsmol/kg. pH values in all samples were measured at different HPS with a Hach H160 handheld meter (Hach Company, Loveland, CO, USA) which was calibrated prior to each round of measurements.

4.4.3. Sperm Viability Analysis

The live:dead sperm cell ratio was determined by flow-cytometric analysis using the LIVE/DEAD Sperm Viability Kit (Invitrogen/Thermo Fisher Scientific Inc.) by S3e™ Cell Sorter (Bio-Rad, Hercules, CA, USA). Prior to running samples, quality control was performed on the S3 Cell Sorter using ProLine™ Universal Calibration Beads. ProSortTM Software was used to generate density plots: FSC (area) *v*. SSC (area) and FSC (area) *v*. FL2 (area). Before measurements, live untreated sperm (negative control) and sperm subjected to repeated storage (positive control) were used to calibrate each fluorescent channel's sensitivity, then thresholds and finally set-up regions of interest. Sperm samples were pre-diluted: 0.5 µL of sperm sample were suspended in 2 mL of 0.9% (*w*/*v*) NaCl solution. Then, 5 µL propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) was added to the sperm solutions, vortexed for a few seconds and incubated for at least 15 min on ice. A minimum of 10,000 spermatozoa were analyzed using a S3e Cell Sorter. The data were processed using ProSort™ software (Bio-Rad, Hercules, CA, USA). The populations with different PI channel intensities were compared for each sample and correlated with membrane damage. Populations with high PI fluorescent signals were considered as dead cells. The percentage of live:dead sperm cells was calculated based on this ratio between populations with low and high PI fluorescence intensity [73].

4.5. Examining Fertilization, Hatching and Larval Malformation Rates during Spermatozoa In Vitro Aging

For the fertilization experiments, pooled in vitro stored milt at different HPS were diluted in Kurokura immobilizing solution (KIS) [35], then pooled fresh eggs (0.10 g, about 70 eggs) from three females at each HPS were inseminated by pipetting 16.45 µL milt at 1, 24 and 96 HPS with 31,250,000 diluted spermatozoa (100 µL milt + 900 µL KIS).

Prior to fertilization at 24 and 96 HPS, pooled fresh milt controls from three males were diluted with the KIS, respectively. Eggs (0.10 g = approximately 0.1 mL, about 70 eggs) were inseminated by pipetting 11.41 µL for the fertilization assays at 24 and 96 HPS with again 31,250,000 diluted spermatozoa (100 µL milt + 900 µL KIS).

Milt was not added directly to the eggs, but the pipette was inserted to the bottom of the 25 mL beaker near the eggs. This procedure was repeated four times, resulting in four beakers being placed on the shaking table (at 21 °C). The shaking table was activated for 1 min (150 rpm), and 2 mL of water was pipetted into each beaker during this period. The fertilized eggs were carefully transferred from the four beakers into four glass Petri

dishes (95 mL; 9 cm in diameter and 1.5 cm in depth). Each Petri dish was filled with dechlorinated water.

Each group of four Petri dishes was kept in four small plastic boxes (13.5 cm × 10 cm × 6.5 cm), filled with dechlorinated water (300 mL) which in turn were placed in one larger plastic box (28 cm × 21.5 cm × 7.5 cm [74]). These boxes were kept in an air-conditioned laboratory at 21 °C. The eggs in each Petri dish were counted as the initial total number of eggs. Dechlorinated water was gently changed in the small plastic boxes at 48 h post-fertilization and non-developing embryos were removed; water was exchanged daily after the eye-stage (48 h post-fertilization) up to hatching. The eye-stage embryos (fertilization) were counted at 48 h post-fertilization and then the hatched larvae and malformed larvae were manually counted directly after hatching over the next 5 days of incubation at 21 °C. The fertilization rate was calculated as the ratio of eye-stage embryos at 48 h post-fertilization to the initial number of eggs per Petri dish. The total hatching rate was calculated as the ratio of all hatched larvae to the initial number of eggs per Petri dish. The malformation rate was calculated as the ratio of malformed larvae (unusual body proportions, e.g., peritoneal or heart edema, irregular body axis and head malformities) to the total number of hatched larvae per Petri dish.

4.6. Whole Genome Bisulfite Sequencing of Sperm

4.6.1. DNA Isolation from Carp Sperm Samples

Samples were processed at 1, 24 and 96 HPS. A volume of 200 µL of each sample was transferred to new sterile microtubes and samples were centrifuged at 17,000× *g* at 4 °C for 15 min twice to eliminate the supernatants (Thermo Fisher Scientific). Pellets were used to isolate the genomic DNA using RNA blue (Top-Bio, Vestec, Czech Republic) following the protocol for DNA isolation.

DNA concentration, protein and salt contamination were determined spectrophotometrically using Nanophotometer Pearl (Implen, Munich, Germany). The quality and integrity of gDNA was further tested by restriction digestion by EcoRI followed by electrophoretic separation and visual inspection of intact gDNA and the corresponding restriction digest product in 1.5% agarose gel.

One µg of total DNA of each sample was sent for WGBS to Admera Health Biopharma Services. The bisulfite conversion was done using Zymo EZ DNA Methylation Gold Kit (Zymo Research, Irvine, CA, USA), and the sequencing library was prepared using Swift Accel-NGS MethylSeq DNA library kit (Swift Biosciences, Ann Arbor, MI, USA) producing a directional sequencing library. The sequencing was done with Illumina PE 150 Cycle (Illumina, San Diego, CA, USA).

The quality of raw sequencing data was verified with fastQC-0.11.5 [75], and sequencing adapters and low-quality reads were removed with Trimmomatic-0.36 [76] using the following parameters: ILLUMINACLIP:seqprimers.txt:2:25:10 LEADING:4 TRAILING:4 SLIDINGWINDOW:4:15 MINLEN:36.

The clean reads were mapped to the common carp genome assembly (accession number GCA_000951615.2) using Bismark Bisulfite Read Mapper v0.22.3 [77] with—score_min value L, 0–0.6. Duplicated reads were removed from the Bismark output files with the Bismark_deduplicate script.

The counts of methylated and non-methylated cytosines were retrieved with the Bismark_methylation_extractor, and a pairwise differential methylation analysis was performed in Defiant v 1.1.9 [78] with minimum differential nucleotide count 4 (-d switch), minimum number of CpN 5 (-CpN switch) and -v switch with a bh argument to print a *p*-value supported by a Benjamini-Hochberg correction for each differentially methylated region (DMR). The remaining options were set to their default values or were not used if the default value was not preset. The records of DMRs were removed from the final count table if their q value was lower than 0.05.

4.6.2. Functional Annotation of Differentially Methylated Regions

Chromosomal co-ordinates in the count tables of DMR were paired with the carp genome annotation file in order to collect the corresponding protein IDs if present. When the protein ID was not available, locus, transcript or gene IDs were used to search for protein ID in GenBank using an in-house BioPython v1.78 script employing the Entrez module. The remaining unannotated DMRs were further manually annotated. Chromosomal sequences of some unannotated DMRs were used to retrieve transcript ID from common carp transcriptomic data if the percent identity of the best sequence hit was above 95%.

All proteins derived from the carp genome assembly, including the ones newly collected by the above procedure, were collectively processed with InterProScan v 5.48–83.0 with the Goterms parameter [79]. The resulting report was parsed to assign GO terms to sequences in this set. A list of all accessions provided a background for functional enrichment of DMRs, whereas lists of DMRs represented the study group for the enrichment analysis.

Functional enrichment was performed by GOATOOLS, v0.8.12 [80] with Benjamini-Hochberg false discovery rate and gene ontology file version 2021-02-01.

4.7. Data Evaluation and Statistical Analysis

The data distribution homogeneity of dispersion was evaluated using the Levene's test. One-way analysis of variance (ANOVA) with an LSD test for post hoc multiple comparisons was used to test for differences among the groups between sperm motility, sperm total concentration, motile sperm concentration, velocity of spermatozoa, percentage of dead and live cells, pH of seminal plasma, osmolality of seminal plasma, fertilization rate, hatching rate and malformation rate.

The DMRs were calculated by Defiant [77] which infers the DMRs using Weighted Welsch expansion hence lending a higher significance to each of the three biological replicates with greater coverage.

Line charts of sperm motility, percentage of live and dead cells, and fertilization, hatching and malformation rates were drawn with mean ± standard deviation of the mean (S.D.). All analyses were performed at a significant level of $p < 0.05$ in R 3.3.2 [81].

5. Conclusions

Significant decreases in the phenotypic characteristics of common carp sperm were detected after aging for 96 h from stripping. Meanwhile, seminal fluid characteristics such as pH and osmolality gradually decreased and increased, respectively. An age-related decrease in fertilization and hatching rates by the aging of common carp sperm was observed. Aging was associated with a dynamic changes of sperm DNA methylation. An increase in mCpG was found after sperm storage at 24 HPS, in comparison to the sperm at 96 HPS whose methylation level dropped to that of freshly collected sperm at 1 HPS.

Our results contribute to the research studying the molecular basis of sperm aging in common carp, which will provide information to maximize reproductive efficiencies in an important aquaculture species. However, further studies elucidating mechanism of regulation of DNA methylation during sperm aging are needed to be able to use this information in increasing the efficiency of carp breeding management.

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Abbreviations

HPS	Hours post stripping
PSA	Post sperm activation
CpG	Cytosine phosphate guanine dinucleotide
KIS	Kurokura immobilizing solution
PI	Propidium iodide
DMR	Differentially methylated region
MBD-seq	Methyl-CpG binding domain sequencing
DNMT	DNA methyltransferase
RAS	Recirculation Aquaculture System
VCL	Curvilinear velocity
VSL	Straight-line velocity
WGBS	whole-genome bisulfite sequencing

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CHAPTER 7

General discussion

The development of protocols for fish artificial reproduction is a key determinant in aquaculture. Aquaculture is not limited to breeding and culture of economically important species such as common carp and European catfish, but considers endangered species that are critically important for biological conservation such as sturgeon (Billard and Lecointre, 2000; Alavi et al., 2012). Moreover, there are fish species considered as biological model organisms that are highly used in evolutionary and biomedical research such as zebrafish. Basically, artificial reproduction is established based on biological features of reproduction that may differ between species according to natural reproductive ecology and behaviour. In artificial reproduction, stimulation of gamete maturation, fertilization, and incubation of eggs are crucial (Alavi et al., 2008; Mylonas et al., 2010).

A simple method for incubation of sterlet eggs (Chapter 2)

Sterlet is one of 27 species of sturgeons that belongs to Acipenseridae. Among sturgeon species, it has been used as a laboratory model to investigate the biology of reproduction in ancient fishes, and to develop sturgeon aquaculture. In Chapter 2**,** a simple but efficient technique was developed to facilitate the production of fertilized eggs and hatching. In that study, successful incubation of sterlet eggs using the tabletop static Petri dish system in incubation boxes (each 300 mL per Petri dish) and the hatchery tank (300 L) with Petri dish system were observed (**Cheng et al., 2020**). Similarly, Petri dishes were used to incubate catfish eggs for assessment of fertility (Okomoda et al., 2018). This method can be used not only for small size eggs (catfish) but also for larger eggs (salmonids) and incubation of embryos till hatching. Neumiller et al. (2017) reported that 10–15 fertilized eggs of landlocked fall chinook salmon (*Oncorhynchus tshawytscha*) incubated in Petri dishes can be successfully hatched with several water changes. Another study showed that Petri dishes can be used very well for the incubation of rainbow trout embryos for a relatively short period from the eyed egg stage to the hatching of larvae without no need for water changes (Barnes and Durben, 2008). One of the advantages of this method is to easily monitor embryos and larval in Petri dishes during the period of experiments, for instance, those aimed at toxicity assessment (Yamaha et al., 2020; Zhang et al., 2021). In this regard, Barnes et al. (2009) successfully studied the effects of *Flavobacterium columnare* inoculation with antibiotic treatments and resident bacteria on rainbow trout eyed-egg survival and external membrane structure by incubating eyed eggs in Petri dishes. This method provides researchers with a valuable opportunity to design multi-factorial studies with various repetitions to test fertilization. Moreover, water consumption is obviously low for Petri dishes. Therefore, the current study supplies more possibilities for the scientific study if sufficient infrastructure with tanks is not available. To establish the tabletop static Petri dish system, only clean Petri dishes, plastic boxes, and functional air conditioners (used to adjust and stabilize required temperature) are needed that are easily accessible for most of the labs.

Egg activation and fertilization in sterlet (Chapter 3)

The biological processes of egg activation and fertilization are important issues in fish reproductive biology. In Chapter 3**,** we investigated the effects of spermatozoa: egg ratio on fertilization in sturgeon (**Linhart et al., 2020a**). To develop artificial reproduction for aquaculture or research proposes, the identification of minimal and optimal spermatozoa:

egg ratio in fertilization that has been previously evaluated for many fish species (Butts et al., 2009; Nynca et al., 2014). A more interesting parameter that may affect fertilization success is the concentration of spermatozoa in the activation medium during fertilization. The current results showed that a very low concentration of spermatozoa (100–1,300 spermatozoa per mL) displaying about 95% motility with curvilinear velocity over 150 m s^{-1} in water as an activation medium could provide effective fertilization. This may be showing a true reflection of the demand for sperm for the fertilization of sterlet eggs. It has been identified that the required sperm concentration during fertilization is species-specific. For example, Ginzburg (1968) reported that in activating water, a spermatozoa concentration of 10^5 per mL is sufficient for fertilization of sturgeon eggs which is consistent with our results (10^2–10^3 spermatozoa per mL; **Linhart et al., 2020a**); however, to achieve good fertilization, a higher number of spermatozoa (10^7–10^9 per mL) was recommended in lake trout (Ginzburg, 1968). In common carp also higher number of spermatozoa ($1.6x10^7$ per mL) in the activation medium was needed to achieve good fertilization success (Linhart et al., 2015).

Short-term storage of sperm in artificial and physiological seminal plasma in freshwater fishes (Chapter 4)

In short-term storage, sperm is usually diluted with its corresponding seminal fluid or with an extender to maintain sperm in a quiescent state. The suspension is then kept at cool temperatures (0–4) for a short period from hours to days. This method represents an important tool in fish farms, for instance in cases where the male broodfish response to hormonal stimulation quicker than females (Alavi et al., 2012). Frequent studies reveal that sperm diluted in an extender show higher motility and fertilizing ability compared to those of undiluted sperm following a short-term storage (Kurokura et al., 1984; Redondo et al., 1991; Rodina et al., 2004). Further examinations show that the extender protects sperm from osmotic damage and contaminants such as urine, and maintains ATP source required for sperm motility (Linhart et al., 2003; Hatef et al., 2011).

In Chapter 4, it was reported that sperm spontaneous movement was prevented by specific artificial seminal plasma (ASP) or immobilizing solution (IS) in zebrafish and European catfish (**Cheng et al., 2021a**; **Linhart et al., 2020b**). Moreover, ASP had positive effects on sperm short-term preservation in these fishes. The use of IS containing 200 mM NaCl, 30 mM Tris-HCl, pH 7 (Linhart et al., 2004) retained the potential for sperm motility at 60% for 14 days in European catfish. Sperm stored in IS for 7 days, produced both fertilization and hatching rates of 80% when spermatozoa: oocyte ratio was 10^5:1. The possible reasons were that sperm was collected by vacuum-pump which reduce the potential of microbial contamination. It is worth noting that the temperature used for sperm storage (0–4) could be also an environmental factor to inhibit the growth of any bacterial contamination. The Tris buffer, although, increases the cell membrane permeability (Irvin et al., 1981) but is without big effects on fish sperm. A number of enzymes are inhibited by Tris, and therefore it should be used with care when studying proteins (Desmarais et al., 2002; Ghalanbor et al., 2008). These are probably the reasons why bacteria colonize stored sperm very slowly, and the "mild" toxicity of the buffer did not affect the sperm of European catfish.

In another study (**Xin et al., 2020**), it was observed significant differences in ions concentrations and osmolality of the seminal plasma between bad-quality sperm showing <20% motility and good-quality sperm showing higher than >80%. However, it was interesting that seminal plasma from good sperm showed the ability to revitalize the bad sperm.

Usually, the ASP or IS imitates the composition of the natural seminal plasma, which is a

mixture of various ions with an osmolality of 230–350 mOsm/kg in teleost fish (Morisawa et al., 1983; Kowalski and Cejko, 2019). The sodium, potassium, magnesium, calcium, and chloride ions have been found in fish seminal plasma (Cosson, 2004; Alavi and Cosson, 2006). It is likely that the existence of these ions is necessary, at least in some species, to retain further activation capacity. For example, a study on European eel (*Anguilla anguilla*) has demonstrated that removing potassium or sodium ions from ASP impairs subsequent motility (Pérez, 2020). The optimal composition of ASP or IS may vary among species (reviewed by Beirão et al., 2019).

Management and proper maintenance of sperm is the basic need to obtain high-quality sperm and essential steps for developing a protocol of successful *in vitro* fertilization. The fertilization trial has been widely used in zebrafish, for instance in toxicological exposure studies (Lombó et al., 2019) and in understanding epigenetics modification (Jiang et al., 2013). To establish an efficient protocol for zebrafish artificial reproduction, fertilization methods, sperm: oocyte ratio, sperm concentration per mL and volume of activation solution were explored and determined (**Cheng et al., 2021a**). The differences between the two fertilization methods that using small Petri dishes and half cryopreservation tubes in zebrafish were discussed hereafter: (1) the shape of the containers; (2) the volume of water used for fertilization (the test tube volume was four times smaller); (3) E400 (130 mM KCl, 50 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-(+)-Glucose, and 30 mM HEPES-KOH, pH 7.9, 400 mmol/kg) with milt diluted 1:10 with water in the test tube and 1:40 in the Petri dish; (4) the osmotic concentration being higher in the test tube than in the Petri dish; and (5) the spermatozoa concentration being higher in the test tube than in the Petri dish. Results showed that implication of 6,000,000 spermatozoa (ratio of spermatozoa: oocyte = 1:60,000) was resulted in higher fertilization compared to 600,000 spermatozoa (spermatozoa: oocyte ratio = 1:6,000) following 1.5 h storage. However, a greater number of spermatozoa were needed in our study compared to the $4x10^4$ fresh zebrafish sperm for fertilizing 20–30 eggs (Hagedorn and Carter, 2011). It was also reported that the most effective parameter that can be created in these two fertilization techniques was sperm concentration. In addition, it was also found that the fertilization rate of aging sperm of European catfish after one-week storage was reduced compared to fresh sperm, however, it can be compensated by increasing the number of spermatozoa to achieve over 80% of fertilization and hatching. Even for fresh sperm, the minimum sperm needed during artificial fertilization is diverse among Cypriniformes, Salmoniformes, Siluriformes, and also between different marine fish species (Beirão et al., 2019).

Practical application of activation solutions for fertilization of aged sperm of common carp (Chapter 5)

There are various parameters affecting artificial reproduction success (Alavi et al., 2008; Fauvel et al., 2010). Parental parameters (such as age and health) and environmental parameters (such as temperature and photoperiod) affect the gametes quality. Also, the contribution of parents to gametes quality is influenced by nutrition, environmental contaminants (such as xenobiotics) and domestication. Finally, technical factors including hormonal stimulation of gametes maturation, methods to collect the gametes, the time between hormonal stimulation and stripping, frequency of gamete striping from an adult, and protocol of artificial fertilization affect the artificial reproduction success. In Chapter 5, it was studied the effects of fertilization medium on the fertility of aged sperm of common carp (**Cheng et al., 2022**). In a hatchery scale, fertilization and hatching rates higher than 80% were obtained when 7-days-stored sperm of carp in artificial seminal plasma was activated

in either hatchery water or Perchec's activation solution containing 45 mM NaCl, 5 mM KCl, 30 mM Tris-HCl; pH 8.0 (Perchec et al., 1996). It has been demonstrated that activation media influence the spermatozoa motility kinetics including the percentage of motile spermatozoa, spermatozoa viability and spermatozoa velocity parameters (Cosson, 2004). In this context, ionic composition and osmolality of the activation medium are key determinants of artificial reproduction success (Fauvel et al., 2010) due to osmolality-dependent spermatozoa motility initiation (Morisawa, 2008; Alavi et al., 2019). The effects of environmental osmolality and ions have been previously studied for many fish species including common carp (Billard et al., 1995), European catfish (Linhart et al., 1997) and zebrafish (Wilson-Leedy et al., 2009). Moreover, stripping or natural spawning of eggs is accompanied by the release of ovarian fluid in fishes that may also change the artificial fertilization environment (Zadmajid et al., 2019). Furthermore, the activation medium also influences eggs ability for fertilization (Żarski et al., 2015). In an activation medium with higher osmolality (200 mOsm kg^{-1}), fresh carp eggs could be fertilized for up to 360 s probably due to the osmolality effects on the duration of motile spermatozoa (Żarski et al., 2015). In our study, we observed that a medium with lower osmolality can activate aging sperm within a short time than higher osmolality (**Cheng et al., 2022**). Overall, the osmolality of the activation medium critically affects fertilization, and it is possible that different osmolality is required to optimize the artificial fertilization of aged sperm compared to fresh sperm to achieve the highest fertilization rate.

Phenotypes changes and DNA methylation levels in aging sperm of common carp (Chapter 6)

Refrigerated short-term storage of sperm is a simple and low-cost method to develop aquaculture management. It has been used in *in vitro* fertilization for laboratory research, hatchery production, and breeding selection proposes (Contreras et al., 2020). However, spermatozoa undergo physiological alternations during the period of storage associated with a decrease in viability, and changes in phenotypic characteristics (such as integrity of plasma membrane, the integrity of the mitochondria and motility kinetics) as well as functional properties of genome material (such as integrity of DNA and methylation levels of DNA) (reviewed by Contreras et al., 2020).

In the current study (**Cheng et al., 2021b**), we found that the time of *in vitro* storage has a considerable impact on the quality of spermatozoa in common carp. Following 24 hours of storage, the motility rate and the number of live spermatozoa reduced significantly resulting in a decrease in fertilizing ability from 90% to 67%. However, velocity parameters of spermatozoa (VCL and VSL) remained unaffected as >50% of spermatozoa exhibited velocity >100 m s^{-1}). The method to store the sperm highly affects spermatozoa motility kinetics, and may depend on species. In common carp, motility rate, progressive motile sperm and curvilinear velocity of sperm (VCL, m s^{-1}) after two-weeks storage in artificial seminal plasma (2 mM $CaCl_2$, 1 mM $MgSO_4$, 40 mM KCl, 100 mM NaCl, 20 mM Tris; pH 7.5 and 310 mOsm kg^{-1}) containing antibiotics were 55%, 25% and 120 m s^{-1} (Cejko et al., 2022). The percentage of spermatozoa motility was 20-40% in rainbow trout following 13 days of storage in a saline solution (0.2% KCl and 0.7% NaCl, DMSO 1%) (Ubilla et al., 2015). In the present study, sperm changes were also investigated under physiological conditions, i.e. without artificial seminal plasma. Interestingly, results showed that only 55% of the original total spermatozoa number per mL remained in the pooled sperm, while 69% of the original total spermatozoa number per mL remained in the sperm from individual males following 96 hours of storage. This may suggest sperm concentration changes after short-term storage. However, this phenomenon

was mostly ignored to re-calculate sperm: egg ratio during fertilization which needs to be paid attention to in further studies.

Along with variations in sperm phenotypes, sperm epigenetics may be changed during the period of storage. Epigenetic changes including DNA methylation have been largely studied in human and mammalian sperm (Donkin and Barrès, 2018). However, little information is available on fish sperm, especially in species that are economically important in aquaculture. One of our studies was aimed at determining the CpG methylation level in fresh and aged sperm of common carp (**Cheng et al., 2021b**). We found that percentage of DNA methylation was increased especially after 24 h storage which was associated with a significant decrease in fertilizing ability. A previous study suggested a relationship between differentially methylated regions and fertilizing ability of sperm in striped bass (Woods et al., 2018). Analysis of DNA methylation in striped bass spermatozoa with the high fertilizing ability and with sub-fertility demonstrated 171 DMRs, which were correlated to fertility. After functional classification analysis, two important genes were identified from WDR3/UTP12 and GPCR families that involve in the formation of the sperm flagella hormonal signalling, and regulation of tissue development, proliferation, and differentiation (Woods et al., 2018). However, in our study, there was no enrichment in either of the research groups that were compared after the performance of gene ontology (GO) enrichment of annotated DMRs. It is worth to note finding many orthologs genes in zebrafish using common carp gene ID cause limitation, due to the lack of a well-annotated genome in common carp. Therefore, it was recommended to annotate the DMRs more thoroughly in the future.

In human sperm, it has been discovered that infertile patients' sperm contained DNAme abnormalities at imprinted loci (Hammoud et al., 2010). In public, most attention is paid to studying the effect of aging on the DNAme landscape of human sperm to develop assisted reproductive technologies (ARTs). It has been observed that global sperm DNA methylation patterns are stable over short periods but increase with age (Jenkins et al., 2013). Sperm DNA methylation mistakes are more frequent in males with aberrant semen parameters (e.g., ICSI candidates) than in normozoospermic controls (Reik and Walter, 2001; Marques et al., 2004). A recent study reported that ART may affect fetal development through epigenetic mechanisms due to both fresh and frozen embryo transfer showing DNA methylation differences (Håberg et al., 2022).

In fish sperm, some studies have investigated the effect of hormonal induction, cryopreservation on DNA methylation and the relationship between DNA methylation and sperm fertility (de Mello et al., 2017; Woods et al., 2018; Herranz-Jusdado et al., 2019; Depincé et al., 2020). Regarding sperm cryopreservation, DNA methylation depends on the type of cryoprotectant (Herranz-Jusdado et al., 2019). Therefore, the knowledge is very poor to understand the association between DNA methylation of sperm and functional properties of sperm that are determinants of fertility. In this context, it is essential to study the affected genomic areas using the Next-Generation Sequencing (NGS) such as the whole-genome bisulfite sequencing (WGBS).

Studying of fish sperm DNA methylation provide us with valuable information to understand the intergenerational and transgenerational inheritance of reproductive phenotypes or disorders (such as diminished sperm motility kinetics and fertility). So far studies on human and animal models suggest that some changes in DNA methylation patterns in males have a negative impact on both reproductive and offspring development (Zheng et al., 2021). In zebrafish, sperm, but not oocyte, DNA methylome is inherited by early embryos (Jiang et al., 2013), and at the same stage of mid-blastula transition, the zygotic genome activation occurs (Kane and Kimmel, 1993). Studies suggest the possibility of transmission of epigenotoxic effects from spermatozoa to offspring after maternal, paternal, and embryonic exposure

in zebrafish (Lombó et al., 2015). In terms of paternal inheritance, adult male exposed to 2,000 g/L BPA has been linked to cardiac abnormalities in progeny up to the F2 generation (Lombó et al., 2015). And it was indicated that these abnormalities were produced by an increase in spermatozoa histone acetylation that was passed down to the F1, notably impacting genes involved in cardiogenesis (*hand2*, *esr2b*, and *kat6a*) (Lombó and Herráez, 2021). Overall, the epigenetic inheritance of reproductive disorders and phenotypic changes in spermatozoa following short-term storage remained to be elucidated in further studies.

Conclusions

In the present thesis that includes seven publications, (a) methods for egg incubation and artificial fertilization of eggs were optimized in sterlet, (b) short-term storage of sperm in artificial and physiological seminal plasma, and optimum fertilization conditions including sperm concentration per egg per mL of activation solution were determined for zebrafish, sterlet and European catfish, (c) various sperm activating solutions were examined to assess sperm fertility in common carp following short-term storage at hatchery scale, and (d) molecular mechanisms of DNA methylation associated with sperm phenotype changes behind aging sperm have been investigated in common carp.

The current results contributed significantly to a better practical implication of sperm in aquaculture of economic fish species (sturgeon, common carp and European catfish) and fish as a biological model (zebrafish), and a better understanding of the basic theology of sperm preservation, which should be beneficial to the development of aquaculture of these species and further mechanism research in sperm function.

The conclusions specifically obtained from the works presented in this thesis were as follows:

- **Chapter 2**
  Similar development of neurulation and embryos undergoing cleavage was achieved when eggs were incubated in Petri dishes with the use of the static tabletop system in the hatchery tank and incubator. Petri dishes in the static table-top system provided a convenient and efficient method to incubate sterlet eggs, but also other species such as common carp and zebrafish.

- **Chapter 3**
  A very low concentration of spermatozoa with excellent motility and velocity after activation in water can provide effective fertilization in the sterlet. Moreover, a larger dilution of sperm and eggs was more suitable for fertilization in sturgeons than has been previously reported.

- **Chapter 4**
  In zebrafish, the most effective way for achieving a high level of fertilization and hatchability was to use a half cryopreservation tube with a precisely defined amount of sperm than Petri dishes.
  Spermatozoa of European catfish stored in immobilizing solution (IS) for two weeks still had 60% motility and high velocity. In addition, sperm can be collected and stored in IS for as long as one week, retaining quality sufficient for fertilization to yield normal fry.

Sterlet sperm quality can be revitalized by storage in good quality of seminal plasma. The fertilization capacity of bad sperm incubated in good seminal plasma can reach levels of fertilizing ability of good quality sperm. Moreover, low ion concentration and osmolality in bad seminal plasma may be a partial cause of lower sperm quality.

- **Chapter 5**
  Fertilization and hatching rate after one week of sperm storage in an ASP (2 mM $CaCl_2$, 1 mM $MgSO_4$, 40 mM KCl, 110 mM NaCl, 20 mM Tris; pH 7.5 and 310 mOsm/kg) were high (78–87%) in activation solutions of hatchery water and/or the Perchec's activation solution (45 mM NaCl, 5 mM KCl, 30 mM Tris, pH 8.5). However, less than 1% sperm motility, fertilization and hatching rate were observed in one week of stored undiluted common carp sperm with similar ASP. Therefore, the fertilization success of one-week-old sperm diluted in a carp ASP at 4 °C with activation solutions confirmed that the stored sperm could be used in common carp aquaculture on a large scale.

- **Chapter 6**
  During the period of sperm storage at 4 °C, sperm phenotype parameters were decreased such as percentage of spermatozoa motility, percentage of fast-motile spermatozoa, spermatozoa velocity, spermatozoa concentration, percentage of live spermatozoa, and pH of seminal plasma, while osmolality of seminal plasma was increased. The spermatozoa methylation level at the CpG sites fluctuates during 96 hours of storage. A more detailed functional analysis of gene-specific differences in the DNA methylation was recommended in the further study when the *C. carpio* genome annotation has been completed in the public databases.

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English summary

Short-term storage of sperm is widely used in fish artificial fertilization in hatchery practice due to its convenience, inexpensive and practical benefits. However, it has been well shown that *in vitro* storage of sperm results in phenotypic changes including morphology and motility kinetics associated with changes at molecular levels, which may influence fertilization success. The main aim of my works was directed toward understanding the effects of sperm quality on fertilization success in some selected freshwater fish species. Firstly, methods for incubation of fertilized eggs were optimized that are suitable for laboratory-based studies. Then, methods were developed for sperm management to achieve high fertilization success in artificial reproduction, when short-term-stored sperm was used. Finally, additional studies were conducted to investigate phenotypic and epigenetic changes in aging sperm following short-term storage. In this context, sperm motility kinetics and DNA methylation levels were analysed which are essential for revealing the mechanisms behind sperm aging.

A technique was developed to supply a simple and efficient method for incubation of eggs in sterlet (*Acipenser ruthenus*) by comparing three different instruments that consisted of placing Petri dishes (a) in the hatchery tank, (b) on a table in a small incubation box in an air-conditioned room or (c) in a large thermostatic incubator box (Chapter 2). The percentage of cleavage, neurulation and hatching rates of embryos and larvae were evaluated. There were no significant differences found among these egg incubation systems in Petri dishes. The experience gained allowed us to practice this method for incubation of eggs of other fish species such as common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) in Petri dishes.

A method was standardized for artificial fertilization of sterlet oocytes (Chapter 3). A series of experiments were conducted to examine the effects of spermatozoa number and volume of activation solution to determine the minimum number of spermatozoa with an optimal volume of activation water for fertilization. The neurulation and hatching rates (%) of embryos were evaluated, and the minimum concentration of spermatozoa per mL of activation water to achieve fertilization success was determined. Results revealed that a total of 50,000 spermatozoa in 8 mL or 32 mL of activation water (6,250 and 1,563 spermatozoa per mL of activation water, respectively) was sufficient for successful fertilization. Also, It was observed that a very low concentration of 100–1,300 spermatozoa per mL in activation water can achieve efficient fertilization if sperm quality is high showing 95% motility with a curvilinear velocity of above 150 m s^{-1}.

In Chapter 4, artificial and physiological seminal plasma were used as extenders to prevent sperm spontaneous movement during the period of storage. *In vitro* artificial reproduction was also optimized in zebrafish with consideration to the identification of an optimum extender for sperm short-term storage, spermatozoa: egg ratio and minimum sperm concentration per mL of activation solution for fertilization. An extender called E400 containing 130 mM KCl, 50 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-(+)-Glucose, and 30 mM HEPES-KOH, pH 7.9 with osmolality of 400 mOsmol/kg was efficient for short-term storage of zebrafish sperm. A test tube with 6,000,000 zebrafish spermatozoa in E400 extender per 100 eggs and 100 L of activation solution has proven to be more successful than using a Petri dish. Sperm of European catfish (*Silurus glanis*) can be collected and stored in immobilization solution for as long as 1 week, retaining quality sufficient for successful fertilization to yield normal fry. It was also found that bad quality of sterlet sperm can be revitalized by incubation with seminal plasma from good sperm. These results help to establish methods for *in vitro* storage of sperm to achieve fertilization success in freshwater fishes.

Activation solution is one of the key factors affecting fertilization success. Different activation solutions were used to test the practical fertilization application efficiency in a

hatchery jar using short-term-stored sperm. Aged sperm was diluted in artificial seminal plasma (Chapter 5). The large-scale artificial fertilization trials indicated that fertilization and hatching rates were high (78–87%) when one-week-stored sperm of common carp in artificial seminal plasma was used after activation in hatchery water and Perchec solution (45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8.0). This study offers a practical basis for the further application of aging sperm in a hatchery.

In Chapter 6, motility kinematics of aged sperm was studied in common carp using a computer-assisted sperm analysis (CASA) system. Accurate assessments of sperm viability, osmolality and pH of seminal plasma were performed to evaluate the effects of short-term storage on sperm functions. Moreover, DNA methylation levels were analysed based on the whole-genome bisulfite sequencing (WGBS) to investigate molecular changes in aging sperm genome. The results showed that aging spermatozoa in common carp has a considerable negative impact on their performance and as well as artificial fertilization success. However, the sperm viability level was much higher than sperm motility, especially after 4 days of sperm storage. Results suggest us, in the future, to improve the environment of aging sperm, which may help to activate the immotile spermatozoa. Additionally, DNA methylation levels at the cytosine-phosphate-guanine (CpG) sites were increased significantly following 24 hours of storage spermatozoa compared to fresh sperm, but then reduced significantly following 96 hours of storage. The results contribute to investigating the sperm phenotype and functional changes in common carp and provide clues for understanding the molecular changes during short-term storage.

Overall, the results of these research improve our knowledge of fish reproduction especially on the management of sperm during short-term storage and enhanced our understanding of the sperm aging process at epigenetic levels. Our data provided the basic information for hatchery applications in practical operation and basic research levels. Further research needs to study changes of DNA methylation in other species, as well as histone modification, and confirmation of transformation possibility of changed spermatozoa DNA methylation into the next generations appear to be worthwhile.

Czech summary

Krátkodobé uchování spermatu v líhňařské praxi ryb je hojně využívanou technikou při umělém oplození, a to díky své výhodnosti, nízké ceně a praktičnosti. Předchozí studie ovšem ukázaly, že spermie uchovávané *in vitro* mohou být poškozeny po stránce morfologické, kinetické i na molekulární úrovni, a to vše může ovlivnit úspěšnost oplození. Hlavním cílem mé práce bylo poznání vlivu kvality spermatu na úspěšnost oplození u některých vybraných druhů sladkovodních ryb. Nejprve byly optimalizovány metody inkubace oplozených jiker, které jsou vhodné pro laboratorní studie. Následně byly vyvinuty metody pro management krátkodobě uchovávaných spermií s cílem dosažení vysoké úspěšnosti oplození při umělé reprodukci. Na závěr byly provedeny další studie zaměřené na zkoumání fenotypových a genomických změn u stárnoucích spermií po krátkodobém uchování. V této souvislosti byla analyzována kinetika motility spermií a úrovně metylace DNA, které jsou zásadní pro odhalení mechanizmů stárnutí spermií.

Byla vyvinuta technika, která poskytuje jednoduchý a účinný způsob inkubace jiker jesetera (*Acipenser ruthenus*) porovnáním tří různých metod. Ty spočívaly v umístění Petriho misek a) v nádrži líhně, b) na stole v malém inkubačním boxu v klimatizované místnosti nebo c) ve velkém termostatickém inkubačním boxu (kapitola 2). Bylo hodnoceno procento rýhování, neurulace a líhnutí embryí a larev. Mezi těmito systémy inkubace jiker v Petriho miskách nebyly nalezeny žádné významné rozdíly. Získané zkušenosti nám umožnily využít inkubaci jiker v Petriho miskách i u kapra obecného (*Cyprinus carpio*) a zebřičky pruhované (*Danio rerio*).

U jesetera malého byla standardizována metoda pro umělé oplození jiker (kapitola 3). Experimenty spočívaly ve zkoumání účinků počtu spermií a objemu aktivačního roztoku na stanovení minimálního počtu spermií pro daný objem aktivační vody pro oplození. Úspěšnost oplození se hodnotila na úrovni neurulace a líhnutí (%) embryí, včetně stanovení minimální koncentrace spermií na mL aktivační vody. Výsledky ukázaly, že k úspěšnému oplození postačuje celkem 50 000 spermií v 8 mL, resp. 32 mL aktivační vody (koncentrace 6 250, resp. 1 563 spermií na mL aktivační vody). Bylo zjištěno, že velmi nízká koncentrace 100–1 300 spermií v mL aktivační vody může zabezpečit účinné oplodnění při použití kvalitního spermatu vykazujícího 95% pohyblivost s křivočarou rychlostí nad 150 μm s^{-1}.

V kapitole 4 byla použita umělá a fyziologická seminální plazma jako ředidlo k zabránění spontánního pohybu spermií během doby uchování. U zebřičky pruhované byl optimalizován způsob osemenění jiker při umělé reprodukci *in vitro* s ohledem na i) identifi kaci optimálního ředidla pro krátkodobé uchování spermatu, ii) poměr počtu spermií na vajíčko a iii) minimální koncentraci spermií na mL aktivačního roztoku. Ředidlo (extender) nazvaný E400 obsahující 130 mM KCl, 50 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-(+)-Glucose, 30 mM HEPES-KOH, pH 7.9 o osmolalitě 400 mOsmol/kg byl účinný při krátkodobém uchování spermií zebřičky pruhované. Zkumavka s přesně definovaným množstvím 6 000 000 spermií zebřičky v extendoru E400 na 100 vajíček a 100 μl aktivačního roztoku se ukázala být úspěšnější než použití Petriho misky. Spermie sumce velkého lze odebrat a skladovat v imobilizačním roztoku po dobu až 1 týdne, přičemž si spermie zachovávají dostatečnou kvalitu pro úspěšné oplodnění a získání normálního plůdku. Bylo rovněž zjištěno, že spermie jesetera malého špatné kvality lze revitalizovat inkubací v seminální plazmě z dobrého spermatu. Tyto výsledky pomohou zavést nové metody uchování spermií *in vitro* při zajištění dostatečné oplozenosti jiker u sladkovodních ryb.

Aktivační roztok je jedním z klíčových faktorů ovlivňujících úspěšnost oplození. Byla testována účinnost různých aktivačních roztoků při oplozování jiker krátkodobě uchovaným spermatem, a to při inkubaci jiker v klasických inkubačních lahvích. Stárnoucí spermie byly naředěné v umělé seminální plazmě (kapitola 5). Rozsáhlé fertilizační pokusy ukázaly, že při

použití spermatu kapra obecného uchovávaného týden v umělé seminální plazmě byla míra oplození jiker a líhnutí larev vysoká (78–87 %), pokud se pro aktivaci gamet použila voda z líhně a roztok Perchec (45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8,0). Výsledky studie nabízejí možnost využití stárnoucích spermií v líhňařské praxi.

V kapitole 6 byla studována kinematika motility zestarlých spermií u kapra obecného pomocí systému počítačové analýzy spermií (CASA). Bylo provedeno přesné hodnocení životaschopnosti spermií, osmolality a pH semenné plazmy, aby bylo možné vyhodnotit vliv krátkodobého uchování *in vitro* na funkčnost spermií. Kromě toho byly analyzovány hladiny methylace DNA na základě celogenomového bisulfitového sekvenování (WGBS). Výsledky ukázaly, že stárnutí u spermií kapra obecného má značně negativní dopad na jejich výkonnost a také na úspěšnost umělého oplodnění. Životaschopnost spermií byla ovšem mnohem vyšší než jejich pohyblivost, a to zejména po 4 dnech uchování. Zlepšení prostředí u stárnoucích spermií by nám tak mohlo pomoci aktivovat již nepohyblivé spermie. Kromě toho byla pozorována významně vyšší hladina methylace DNA v místech cytosin-fosfát-guanin (CpG) po 24 hodinách uchování spermatu ve srovnání s čerstvým spermatem. Ta se ale po 96 hodinách uchování opět snížila. Výsledky přispívají ke zkoumání fenotypu a funkčních změn spermií u kapra obecného a poskytují vodítka pro pochopení molekulárních změn během krátkodobého uchování.

Výsledky získané řešením této práce rozšířily naše znalosti o reprodukci ryb, umožnily upravit management se spermatem během krátkodobého uchování a pomohly nám lépe chápat proces stárnutí spermií na epigenetické úrovni. Naše data poskytla základní informace pro aplikace v líhních, tedy v praktickém provozu a na úrovni základního výzkumu. Zdá se, že další výzkum změn metylace DNA a modifikace histonů u jiných druhů ryb, včetně potvrzení možnosti transformace změněné metylace DNA u spermií a její přenos do další generace může být užitečný.

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List of publications

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Sun, H.Y., Yao, J.L., **Cheng, Y.**, Chen, Y.F., 2016. Length-weight relationships of six fish species from the Lancang River, China. Journal of Applied Ichthyology 32, 509–510. (IF 2016 = 0.845)

Abstracts and conference proceedings

Cheng, Y., Xin, M.M., Gela, D., Rodina, M., Tučková, V., Kašpar, V., Siddique, M.A.M., Shelton, W.L., Linhart, O., 2019. Techniques for optimization of sterlet (*Acipenser ruthenus*) egg incubation for laboratory use. In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, 2nd–6th September 2019, Rennes, France, p. 46.

Linhart, O., **Cheng, Y.**, Rodina, M., Tuckova, V., Kaspar, V., Xin, M.M., 2019. Hundreds of spermatozoa are sufficient for fertilization of starlet (*Acipenser ruthenus*) eggs. In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, 2nd–6th September 2019, Rennes, France, p. 64.

Rahi, D., Dzyuba, B., Xin, M.M., **Cheng, Y.**, Dzyuba, V., 2019. Inhibitory analysis of energy supplying pathways of motile and immotile spermatozoa in Siberian sturgeon (*Acipenser baerii*). In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, 2nd–6th September 2019, Rennes, France, p. 63.

Training and supervision plan during study

Name	Yu Cheng
Research department	2018–2022 – Laboratory of Reproductive Physiology of FFPW
Supervisor	Prof. Otomar Linhart
Period	25th October 2018 until 14th September 2022

Ph.D. courses	**Year**
Basic of scientific communication	2019
Pond aquaculture	2020
Fish reproduction	2019
Applied hydrobiology	2019
Ichthyology and fish taxonomy	2020
English language	2021

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2019 2020 2021 2022

International conferences	**Year**
Cheng, Y., Xin, M.M., Gela, D., Rodina, M., Tučková, V., Kašpar, V., Siddique, M.A.M., Shelton, W.L., Linhart, O., 2019. Techniques for optimization of sterlet (*Acipenser ruthenus*) egg incubation for laboratory use. In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, 2nd–6th September 2019, Rennes, France, p. 46.	2019
Linhart, O., **Cheng, Y.**, Rodina, M., Tuckova, V., Kaspar, V., Xin, M.M., 2019. Hundreds of spermatozoa are sufficient for fertilization of starlet (*Acipenser ruthenus*) eggs. In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, 2nd–6th September 2019, Rennes, France, p. 64.	2019
Rahi, D., Dzyuba, B., Xin, M.M., **Cheng, Y.**, Dzyuba, V., 2019. Inhibitory analysis of energy supplying pathways of motile and immotile spermatozoa in Siberian sturgeon (*Acipenser baerii*). In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, 2nd–6th September 2019, Rennes, France, p. 63.	2019

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Assoc. Prof. Mariola Dietrich, Gamete and Embryo Biology, Polish Academy of Sciences in Olsztyn, Poland (2 months, proteomics and sperm cryopreservation)	2021
Dr. Catherine Labbé, Fish Physiology and Genomics, INRAE, Rennes, France (2 months, DNA methylation and bioinformatics)	2022

Pedagogical activities	**Year**
• Leading of project entitled "Short-term storage of sperm in common carp (*Cyprinus carpio*)" at summer school	2021
• Anouncing the project entitled "Short-term storage of sperm in some freshwater fish species" at summer school	2020
• Training of students in Laboratory training on sperm motility analysis, DNA and RNA isolation in range of 90 teaching hours	2021

Projects I got in Czech Republic	**Year**
Two-year individual GAJU project-the Grant Agency of the University of South Bohemia (No. 037/2020/Z), Principal Investigator (396,000 CZK in total) – Yu Cheng, Title: Epigenetic modifications and functional properties associated with common carp (*Cyprinus carpio* L.) sperm aging	2020–2021

Curriculum vitae

PERSONAL INFORMATION

Name: Yu
Surname: Cheng
Title: M.Sc.
Born: 10th October, 1991, China
Nationality: Chinese
Languages: English (B2 level – IELTS certificate), Chinese
Contact: ycheng@frov.jcu.cz; ihbchengyu@gmail.com

RESEARCH INTEREST

Preservation of gametes, Artificial fertilization, Evaluation quantity and quality of spermatozoa, DNA methylation in spermatozoa, Sperm histone modification, Proteomics analysis

EDUCATION

2018 – present Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2015–2018 M.Sc., Institute of Hydrobiology, Chinese Academy of Sciences
2011–2015 B.Sc., College of Animal Science and Technology, Shandong Agricultural University, China
2011–2015 B.Sc., College of Economics and Management, Shandong Agricultural University, China

Ph.D. COURSES

Pond aquaculture, Fish reproduction, Applied hydrobiology, Basics of scientific communication, Ichthyology and fish taxonomy, Czech language, English language

TRAINING

24th/06–28th/06 2019 EuPA Summer school on Post-Translational Modifications, from sample preparation to dedicated data interpretation methods and tools, Sète, France.

RESPONSIBLE LEADER OF PROJECTS

International summer school 2021: Short-term storage of sperm in common carp (*Cyprinus carpio*).

RESEARCH STAY AND COLLABORATIONS

11–12. 2021 prof. Andrzej Ciereszko and Asst. Prof. Mariola Dietrich, Gamete and Embryo Biology, Polish Academy of Sciences in Olsztyn, Poland (2 months, proteomics and sperm cryopreservation)

1–3. 2022 Dr. Catherine Labbé, Fish Physiology and Genomics, INRAE, Rennes, France (2 months, DNA methylation and bioinformatics)